Health and Disease Status of Sea Turtles in Western Australia

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"In the end, we will conserve only what we love; we will love only what we understand; and we will understand only what we are taught."

Baba Dioum, Senegalese Conservationist

Thesis Declaration:

I Erina J Young verify that in submitting this thesis;

the thesis is my own account of the research conducted by me, except where other sources are fully acknowledged in the appropriate format,

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that all necessary ethics and safety approvals were obtained, including their relevant approval or permit numbers, as appropriate.

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The content in this thesis was developed by the Candidate with advice from their supervisory panel. The following individuals contributed to the thesis.

Contributor	Contribution (%)	Concept Development	Data Collection	Data Analyses	Drafting of Chapters
Dr Erina Young	75	X	X	X	X
Dr Rebecca Vaughan-Higgins	10	X		X	X
Prof. Kris Warren	5	X			X
Dr Nahiid Stephens	5	X		X	
Dr Lian Yeap	5	X			X

Contribution indicates the total involvement the individual has had in the creation of the thesis. Placing an 'X' in the remaining boxes indicates what aspect(s) of the thesis each individual engaged in.

I would also like to acknowledge Dr Scott Whiting as an external collaborator from Department of Biodiversity, Conservation and Attractions and for his role regarding concept development and data collection.

By signing this document, the Candidate and the Principal Supervisor acknowledge that the above information is accurate and has been agreed to by all other contributors.

Candidate **Principal Supervisor**

Abstract:

The current state of sea turtle health in the Indian Ocean is largely unknown, especially for the endemic flatback turtle (*Natator depressus*) which is listed as 'vulnerable' in Western Australia (WA) and 'data deficient' globally. Anecdotally, the causes of illness, injury, and death in Western Australian turtles are comparable to those in other parts of Australia and the world (e.g., spirorchiidiasis, fibropapillomatosis, and marine debris interaction) but scientific studies to validate these reports are particularly limited in this region. To address these knowledge gaps, causes of both live and dead turtle strandings in WA were investigated through an array of veterinary diagnostic techniques including necropsy, clinical pathology, diagnostic imaging, histopathology, parasitology, microbiology, toxicology, and molecular analyses. Health assessments were conducted on live animals to determine baseline levels of health and disease for specific populations, predominately nesting and foraging flatback turtles.

Through these health and disease investigations, baselines were developed, along with the discovery of new diseases in flatback turtles including a novel haemoparasite, *Haemocystidium* spp., occurring specifically in the foraging life stage; a potentially emerging zoonotic bacterium, *Streptococcus iniae* associated with a multi-species mass mortality event involving post-hatchlings; as well as spirorchiidiasis, previously unreported in this species. Other unusual and emerging diseases were also reported in sea turtles in this study, including microsporidial myopathy, salt gland adenitis, gout, and pseudogout.

In this study, natural disease-related causes of mortality occurred more frequently than direct anthropogenic causes, with parasitoses the most frequently occurring natural disease. Spirorchiidiaisis was the most common cause of mortality (32.0%) with a prevalence of 93.2% in turtles susceptible to the disease (i.e., excluding the post-hatchling life stage). The next most common cause of mortality was unknown (17.3%), followed by trauma (13.3%), endoparasitosis (10.7%), infectious disease (6.7%), and pneumonia (6.7%), with the remaining mortality categories each

accounting for less than 5% of cases (including systemic inflammation, osmoregulatory disorder, gastrointestinal impaction, gastrointestinal foreign body, fibropapillomatosis, and metabolic disorder).

We developed the first flatback turtle reference intervals (RIs) in Reference Value Advisor (RefVal v2.1) following the American Society of Veterinary Clinical Pathology (ASVCP) guidelines. We found flatback turtle RIs were generally similar to other published sea turtle RIs and reference values (RVs) but detected significant differences in our study for the various boundary conditions including life stage (nesting or foraging), as well for measurement methodology (field or laboratory tests), justifying the establishment of separate RIs/RVs for nesting and foraging flatbacks, and for field and laboratory techniques.

This study was the first sea turtle health and disease investigation in WA and the eastern Indian Ocean to offer broader insights into sea turtle health and disease status on a regional scale. These essential baselines provided a number of crucial functions which include serving as a reference point for future studies to monitor changes in population health and disease levels. Specifically, these baseline data will be useful for future comparative studies of the same population where changes are an indication of a changing environment. The blood RIs can be used for disease diagnosis, monitoring progress and assessing prognosis of clinical flatback turtle cases in rehabilitation. Considering that diseases in the marine environment are predicted to rise with increasing anthropogenic pressures, detection of new and emerging diseases is of significance to the global knowledge of sea turtle diseases; and for understanding and mitigating disease threats to sea turtle populations. Finally, this study provided a framework to integrate health into future conservation management decisions to ensure the long-term survival of sea turtles.

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Western Australia. *38th Annual Symposium on Sea Turtle Biology and Conservation, Kobe, Japan, 18-23 February 2018.*

Media reports:

Young, E. 2017. Perth turtle death sparks call for action on plastic pollution. *Conservation Council of Western Australia*. Media release 10 June 2017.

Zimmerman, J. Careless humans kill Tina the turtle with plastic litter. *The West Australian,* 10 June 2017.

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Glossary of abbreviations, acronyms and scientific names

°C	degrees Celsius
µ	micro
µl	microlitre
µmol	micromole
4WD	four-wheel drive
ADH	arginine dihydrolase
AGRF	Australian Genome Research Facility
ALP	alkaline phosphatase
ALT	alanine aminotransferase
amniote	animals with amnion (embryonic membrane) includes mammals, birds and reptiles
ANOVA	Analysis of variance
arribada	mass nesting of sea turtles
ASP	amnesic shellfish poisoning
AST	aspartate transaminase
ASVCP	American Association of Veterinary Clinical Pathology
BCI	body condition index
BCS	body condition score
BIPS	barium impregnated polyethylene spheres
BLAST	Basic Local Alignment Search Tool
BUN	blood urea nitrogen
Ca: P	calcium: phosphorus ratio
carapace	upper (dorsal) shell of a turtle
Caretta caretta	loggerhead turtle
CBC	complete blood count
CCL	curved carapace length
CCW	curved carapace width

cercariae	larval stage of trematode
Chelonia mydas	green turtle
chelonian	reptiles of the order Testudine includes sea turtles, freshwater turtles and tortoises
cheloniid	all extant sea turtle species in the Cheloniidae family except the leatherback
ChHV5	chelonid herpesvirus 5
CI	confidence interval
CK	creatine kinase
CLSI	Clinical Laboratory and Standards Institute
cm	centimetre
costal scutes	scutes of the upper shell overlying the ribs
CT	carapace to tail
CT	computed tomography
D	decomposition score
DBCA	Department of Biodiversity, Conservation and Attractions
DCS	decompression sickness
dermal bone	intramembranous ossification & shell growth centres (also called osteoderm)
Dermochelys coriacea	leatherback turtle
DNA	deoxyribonucleic acid
DPIRD(-DLS)	Department of Primary Industries and Resource Management (Diagnostic Laboratory Services)
DSP	diarrheic shellfish poisoning
ELISA	enzyme-linked immunosorbent assay
epibiota	communities of organisms on external surfaces (also known as epibionts)
Eretmochelys imbricata	hawksbill turtle
esky	portable cooler or ice box
faveoli	alveoli (air sacs) found in reptiles (also known as ediculi)
Fl-ox	fluoride oxalate
forager	foraging turtle

FP	fibropapillomatosis
FTA	Flinders Technology Associates (filter paper for genetic samples)
g	gram
g	gravitational force
G	gauge
ghost net	abandoned, lost or discarded fishing net
GI	gastrointestinal
GLDH	glutamate dehydrogenase
H&E	haematoxylin and eosin
H: L	heterophil: lymphocyte ratio
hb	haemoglobin
HCl	hydrochloric acid
HCT	haematocrit
HSD	honestly significant difference
HW	head width
ID	identification
IFCC	International Federation of Clinical Chemistry
IHC	immunohistochemistry
IM	intramuscular
in	inch
inframarginal scutes	scutes on the bridge of the ventral shell
internesting	period between clutches
IQR	interquartile range
IRS	infrequent restriction site
i-STAT	portable point of care blood analyser
IUCN	International Union for the Conservation of Nature
IV	intravenous
kg	kilogram

km	kilometre
KOH	potassium hydroxide
L	litre
lacrimal glands	salt glands (maintain homeostasis by excreting salt)
LDC	lysine decarboxylase
LDH	lactate dehydrogenase
Lepidochelys olivacea	olive ridley turtle
Li-Hep	lithium heparin
m	metre
M	mole
MALDI-TOF	Matrix-Assisted Laser Desorption Ionisation Time-Of-Flight
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
melanomacrophage	pigmented phagocytes found in poikilotherms
mg	milligram
miracidia	free-living motile stage of trematode
ml	millilitres
mm	millimetres
mmol	millimole
n	sample size
nally tub	plastic box made from heavy duty polypropylene
NATA	National Association of Testing Authorities
Natator depressus	flatback turtle
NBF	non-buffered formalin
neritic	of, relating to, or inhabiting shallow waters over the continental shelf
nester	nesting turtle
NSP	neurotoxic shellfish poisoning

NT	Northern Territory
nurdle	small plastic pellets (building blocks of plastic products)
NWS	North West Shelf
ODC	ornithine decarboxylase
ONPG	ortho-nitrophenyl-β-galactosidase
ontogenetic shift	change of diet or habitat
OR	odds ratio
oviposition	term used to describe the laying of eggs
p	p-value or probability
PAAS	Periodic Acid-Ammoniacal Silver
PAS	Periodic Acid-Schiff
pCO_2	carbon dioxide partial pressure
PCR	polymerase chain reaction
PCV	packed cell volume
plastron	lower (ventral) shell of a turtle
poikilothermic	unable regulate body temperature, also known as ectothermic
pre-frontal scales	scales on the anterior head overlying the prefrontal bone
PSP	paralytic shellfish poisoning
PT	plastron to tail
PZVD	Perth Zoo Veterinary Department
q	every
Rathke's gland	a gland found in some sea turtle species of unknown function
RBC(C)	red blood cell (count)
remigrant	previously marked turtle which returns in a following nesting season
rhamphotheca	horny keratinised beak
RI	reference interval
RMU	regional management units
RNA	ribonucleic acid

rpm	revolutions per minute
RV	reference value
scale	thickened keratinised areas of skin (epidermal-dermal unit) on head, neck & limbs
SCL	straight carapace length
scute	plate-like scales on the shell
SD	standard deviation
SD	standard depth
SI	International System of Units
sp.	species (singular)
spp.	species (plural)
tCO_2	total carbon dioxide
TED	turtle exclusion device
testudine	reptiles of the order Testudine including sea turtles, freshwater turtles and tortoises
the lost years	first 5-10 years of a sea turtle's life about which very little is known
TPS	total plasma solids
TRD	turtle restraining device
trypanorhynch	cestode found in sea turtles as a metacestode stage (cyst)
TSD	temperature sex determination
TWCC	total white cell count
U	units
UV	ultraviolet
V	volts
VT	vent to tail
WA	Western Australia
WAStD	Western Australian Stranding Database
WBC	white blood cell
WT	study's ID prefix for necropsied turtles
WTR	study's ID prefix for baseline health turtles

ZIMS	Zoological Information Management System
ZN	Ziehl-Neelsen
ZR1	basic zoo reptile blood profile
ZR2	standard zoo reptile blood profile

CHAPTER 1

Introduction

1.1 Importance of health in sea turtle conservation

Sea turtles play an integral role in the health of many ecosystems (Pritchard 1997), and likewise the environment has a major influence on the health and normal physiological function of sea turtles. Sea turtles act as bioengineers of foraging habitats (e.g., influencing seagrass community structure and nutrient cycles); hosts (e.g., parasites, epibionts, and pathogens); as components of food webs as both predators and prey; and as suppliers of energy and nutrients into beaches through their eggs (Bjorndal 1997, Jackson et al. 2001, Bjorndal and Jackson 2003, Lovich et al. 2018). However, anthropogenic impacts on the environment are threatening sea turtles' capacity to perform these vital ecological functions and present major risks to their health.

Sea turtles are frequently referred to as sentinels of environmental health, due to their high site-fidelity and longevity (Aguirre and Lutz 2004). As co-inhabitants with humans of coastal environments, their vulnerability to anthropogenic environmental threats is increased; therefore, as indicators of environmental health, their health status often has relevance to human public health (Aguirre and Tabor 2004). The inter-connectedness between human, animal, and the environmental health is recognised by both the One Health and Conservation Medicine disciplines (Schwabe 1964, Kock 1996). While the One Health approach is anthropocentric and strongly focused on improving human health, Conservation Medicine strives to understand these inter-relationships and challenges to wildlife health (both infectious and non-infectious agents) in a changing global environment with the ultimate aim of biodiversity conservation (Daszak et al. 2000, Deem et al. 2001). Here, veterinary specialists are trained specifically in health investigation and disease diagnosis, and particularly well-placed for integration into these transdisciplinary, collaborative health-based projects (Deem and Harris 2017), including to support the conservation of sea turtles and other wildlife.

When considering the importance of health in sea turtle conservation, it is useful to provide an example of how a Conservation Medicine approach is of value for integrating wildlife and environmental health into marine wildlife conservation. Here, the sea otter (*Enhydra lutris*)

is a well-studied example demonstrating the application of Conservation Medicine to identify the relationships among environmental health and the health and population dynamics of a threatened marine vertebrate. Briefly, sea otters are considered keystone species, playing a pivotal role in the health of their ecosystems by maintaining species abundance and diversity, mainly by preying on sea urchins (Jessup et al. 2004). Like sea turtles, otters are a sentinel species; showing high site fidelity, a tendency for bioaccumulation of toxins, and a particular sensitivity to environmental pollution, as evidenced by their high mortality rates from the *Exxon Valdez* oil spill (Bowyer et al. 2003, Garshelis and Johnson 2013). Through long-term disease and mortality research, annual population declines at some locations were shown to be due to increased mortality rather than decreased recruitment (Jessup et al. 2004). These investigations found that a number of novel and emerging infectious diseases (e.g., *Toxoplasma gondii*) and non-infectious diseases (e.g., domoic acid toxicity) were responsible for, or contributed to the majority of sea otter deaths (Kreuder et al. 2003). Furthermore, anthropogenic causes were identified as the sources, including terrestrially-derived domesticated (and invasive) animals such as *T. gondii* oocysts in cat faeces; and excess nutrients and nitrogenous wastes from sewage spills and agricultural run-off (Jessup et al. 2007). In general, understanding the causes of wildlife morbidity and mortality allows wildlife managers to implement management practices to reduce key risks (e.g., implementing feral cat management to limit the reservoir and spread of protozoal parasites), which improves the health of the marine environment and the health and conservation of the wildlife species (Jessup et al. 2004).

The above sea otter example demonstrates how Conservation Medicine may be applied and the value of wildlife health in the context of sea turtle conservation. Similar to other wildlife, most threats to the health and survival of sea turtles are human-induced (Lutcavage et al. 1997, Wallace et al. 2011, Scheelings 2015, Witherington 2017, Dias et al. 2019, Taylor-Brown et al. 2019, Harting et al. 2021). Anthropogenic impacts can cause disease directly such as vessel strike, or indirectly through many complex interacting factors, where ‘disease’ is used to refer to “any impairment that interferes with or modifies performance of natural function” (Wobeser 1981). Here, this definition is extended to include direct anthropogenic impacts such as trauma, toxins, and marine debris interactions. Indirect

anthropogenic health threats can result in both subclinical disease (affecting fitness, reproduction, and survival), such as endocrine disruptor effects of low-level long-term exposure affecting reproduction; or clinical disease, such as the effects of pollution on the depression of immune function, thereby increasing susceptibility to disease outbreaks (Deem et al. 2001, Deem and Harris 2017). The field of wildlife health examines infectious and non-infectious agents and their often complex and interacting relationships; which may result in wildlife disease (Wobeser 2006).

In sea turtle health and disease investigations, baseline data are an important tool for: monitoring population health and trends; developing prognostic indicators for rehabilitated sea turtles; refining disease epidemiology; identifying newly emerging diseases and threat impacts. Given the threat of disease on sea turtle populations is frequently unknown (Commonwealth of Australia 2017b), undertaking disease investigations is particularly urgent. While no sea turtle stocks are at imminent risk of population collapse by known diseases or pathogens, investigations over a range of spatial scales (e.g., regional, global) are required to improve our understanding of disease in sea turtles. Without baselines for causes of sea turtle strandings as well as baseline health investigations, the current or future health status of these transboundary, long-lived threatened migratory species cannot be accurately assessed (Hamann et al. 2010, Wallace et al. 2011, Commonwealth of Australia 2017b). There has not been a time more prudent than the present to develop health baselines considering the close association of health and threats, along with the increasing stressors sea turtles are facing in a changing world. It is therefore timely for health and disease investigations to be discussed.

1.2 Health and disease investigations

Despite a significant increase in sea turtle physiology and biology research, there are fundamental knowledge gaps that inhibit our ability to confidently perform a health assessment that reflects the health status of an individual or population (Herbst and Jacobson 2003). There have been notable advances in this area, particularly since a global, multi-national multidisciplinary assessment in 2010, where the question ‘what constitutes

a normal healthy turtle?' was identified as an area for further research (Hamann et al. 2010, Flint 2013). Despite several knowledge gaps having been addressed such as baseline blood reference intervals (RIs) for many populations, there still remains a paucity of information in relation to some aspects of sea turtle health. This includes identification of subclinical disease, suitability for release based on blood profile (Rees et al. 2016, March et al. 2018), and the role of environmental factors in disease, which remains largely unknown. When considering the innumerable possible interacting environmental factors linked to disease expression in sea turtles, disease processes and reaching a definitive diagnosis are understandably complex. Despite this, standardised clinical health assessments that integrate physiological and ecological knowledge are integral to baseline health surveys and disease investigations (Flint et al. 2010b, Kophamel et al. 2021). Clinical health assessments involve a clinical examination, including assessment of body condition, identification of any apparent abnormalities including neurological deficits combined with the collection of other biometric data, for example species, sex, and size (Flint et al. 2010b). While baseline health surveys evaluate normal and abnormal ranges in a population with the understanding that there are expected to be detectable low levels of disease (e.g., 5%) within populations (Deem and Harris 2017), disease investigations aim to understand the disease process (Herbst and Jacobson 2003). These complementary studies are recommended to be undertaken in collaboration, to improve understanding of sea turtle health and disease (Flint 2013).

Aside from clinical examination, blood sampling is generally included at initial health assessment, because haematology and biochemistry provide a good indication of systemic health (Herbst and Jacobson 2003). Normal RIs have been developed from 'clinically healthy' individuals for many sea turtle species, and once established have important applications at both individual and population levels (Flint et al. 2010b). Providing these RIs are calculated using statistically sound methods, values can be utilised to identify overall health and ill-health of individuals; monitor temporal trends and compare the health of populations; and monitor the progress of sea turtles undergoing rehabilitation to assess suitability for release (Deem et al. 2009, Flint et al. 2010b, Kelly et al. 2015). Some studies have shown specific haematological and biochemical profiles are indicative of the sea

turtle's health status and can predict chance of survival (Keller et al. 2012, Stacy et al. 2013, Li et al. 2015). For example, increased potassium, high pCO_2, and low pH, which are likely indicative of metabolic and respiratory acidosis, have been associated with reduced survival rates, with larger deviations related to increased risk of mortality.

As ectotherms, the influence of both intrinsic (e.g., species, sex, age, size, and physiologic status) and extrinsic factors (e.g., season, habitat, epibiota load, capture method, and other environmental conditions) affecting sea turtle blood parameters requires careful consideration in assessing reference values (RVs) (Flint et al. 2010b, Flint et al. 2010c, Campbell 2012, 2014, Kelly et al. 2015). Variation in blood collection methods, sample handling, and processing techniques can also have significant impacts on RVs (Campbell 2014). Furthermore, chelonians are also reported to have a greater variation in plasma biochemistry values between individuals in a species, due to physiological regulation in reptiles, which maintain normal values over a wider range compared to mammal and avian species (Sposato et al. 2002).

In addition to these intrinsic and extrinsic factors, potential inaccuracies with the case definition of 'clinically healthy', as well as analytical methods and even the selected analytes make interpreting normal parameters problematic. Flint et al. (2010b) defined 'clinically healthy', as a sea turtle: in good body condition; with no abnormalities detected on clinical examination; and no evidence or history of fibropapillomas. In this same study, haematologic and biochemical results were not significantly different for unhealthy and healthy loggerhead turtles, generating the possibility that not all unhealthy loggerhead turtles were excluded from this definition. Disease not reflected by haematological or biochemical changes is another potential explanation for difficulties in defining 'clinically healthy', i.e., the animal has subclinical disease and is either in the latent period, or the disease is not detectable by conventional methods. Traditionally, RIs have been based on analytes relevant to humans or domestic animals which may or may not be applicable to reptiles (Herbst and Jacobson 2003), with many analytes lacking sensitivity and specificity in this group (Stacy and Innis 2017). Additionally, certain analytical methods have yet to be validated in reptiles (Campbell 2014).

Although disease is implicated as a major contributor to sea turtle mortality, the causes of sea turtle deaths are largely unknown, frequently because the turtle is chronically unwell and suffering from a of myriad of diseases, highlighting the significance of disease investigations (Flint et al. 2010d) and the difficulty in attributing proportionate blame to any single factor or aetiology. These studies aim to identify the cause(s) of disease and mortality, and further characterise the pathogenesis and pathophysiology (Herbst and Jacobson 2003). This baseline data are essential to determine disease prevalence and distribution, assess population disease status and identify newly emerging diseases, and will in turn assist conservation management decision making (Flint et al. 2009b).

Disease investigations of free-ranging sea turtles are often opportunistic and isolated, reflecting the sporadic nature of individual cases or mass events. Investigations of mass events are often prioritised due to their population impact, however sporadic cases provide valuable opportunities to increase our understanding of background disease and detect newly emerging diseases (Herbst and Jacobson 2003). Together, these cases represent an estimation of disease levels and mortality rates as the sea turtles salvaged provide an index of actual numbers (Herbst and Jacobson 2003, Flint et al. 2015a). Other potential limitations in assessing sea turtle mortality include unreported stranded sea turtles, strandings occurring on remote and unpatrolled beaches, at-sea mortalities, or turtles found in an advanced state of decomposition and no longer suitable for necropsy (Dobbs 2001). Mortality is likely to be grossly underestimated from fisheries bycatch and drowning, as sea turtles will often have no external marks (Barco et al. 2016). Finally, conducting sea turtle research is expensive and can also be limited by funding, resource and logistical constraints (Flint et al. 2015a).

The few studies that have examined relationships between ante-mortem and post-mortem findings, and risk factors or RVs and disease, have found poor correlation between clinical signs and necropsy findings (Flint et al. 2010d). This is not surprising due to the stoic nature of reptiles and subtlety of clinical signs. Risk factors associated with being ‘clinically unhealthy’ included size (smaller immature and mature sea turtles are more at risk of being diseased) and plastron epibiont load (turtles with >20 individual epibionts adhered to

plastron are at increased risk of being unhealthy) (Flint et al. 2010c). Diseased sea turtles (including stranded individuals) have demonstrated a lower haematocrit and total protein (negative correlation) in comparison to healthy animals (Adnyana et al. 1997b, Whiting et al. 2007a, Deem et al. 2009). However, anaemia and hypoproteinaemia may be masked by concurrent dehydration, which commonly effects diseased turtles, causing haemoconcentration and hyperproteinaemia (Norton et al. 2017).

Despite a recent increase in health and disease investigation, there are still fundamental knowledge gaps (Herbst and Jacobson 2003). Establishment of robust RIs have been highlighted as a priority for sea turtle conservation and management for many years (Aguirre and Balazs 2000). Development of standardised approaches to disease investigations, necropsies, and RVs has improved health and disease studies and allowed for the comparison of studies (Flint et al. 2009b). While systematic and standardised data collection are a good starting place, more research is required to assess the clinical relevance of collected data. Furthermore, as we develop new ways to measure health we can continue to modify and refine our methods (Flint et al. 2010c).

The following sections provide background information on the biology and ecology, distribution, conservation status and threats; the fundamental foundations for understanding of sea turtle health and disease.

1.3 Evolution, biology and ecology

Chelonians are reptiles of the order Testudines and include land tortoises, freshwater or aquatic turtles (sometimes referred to as terrapins), and sea or marine turtles. Only freshwater turtles and sea turtles are found in Australia, and they can be differentiated by their different habitats (e.g., sea turtles are almost entirely marine) and their anatomy, for example, limbs adapted for swimming with most freshwater turtles having webbed feet with nails, while sea turtle limbs are modified into flippers. Conversely land tortoises have adapted to a wholly terrestrial lifestyle which includes limb modifications for walking.

The predominant distinguishing feature of chelonians is the shell, consisting of an upper carapace (a composite of expanded ribs, vertebrae, and dermal bones) and lower plastron (derived from shoulder girdle and rib elements) connected by bony bridges (Wyneken 2001). Other defining characteristics of chelonians include:

- *appendicular skeleton (shoulder and pelvic girdles) located internally within shell (rib derivative)* (Figure 1.1)*;*
- *teeth replaced with a horny keratinised beak or rhamphothecae (except the leatherback turtle, Dermochelys coriacea)*;
- *four-chambered heart* (Mettee et al. 2017, Rodriguez et al. 2018).

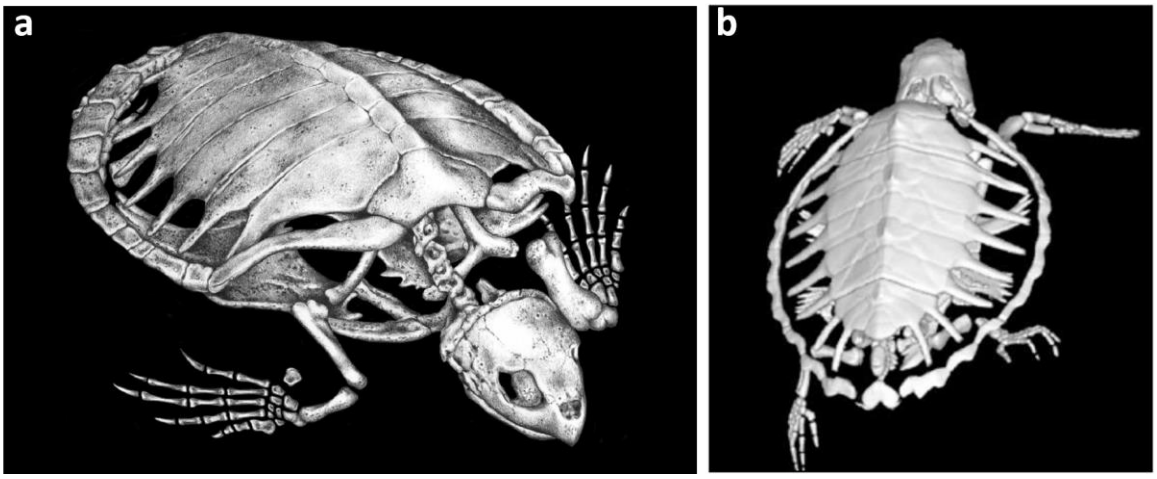

Figure 1.1 Computed Tomography (CT) scans of sea turtle skeleton demonstrating (a) Shoulder location within the shell, and (b) Formation of the bony carapace (Wyneken 2001).

While the origin of sea turtles remains highly contentious (Li et al. 2008, Reisz and Head 2008, Wang et al. 2013, Li et al. 2018), current fossil evidence suggests turtles were one of the earliest reptile groups to diverge (~240 million years ago) (Wellehan 2014, Schoch and Sues 2015, Simões et al. 2018). These first turtles diversified (Hirayama 1998) to exploit the environment, and despite some modifications (Reisz and Head 2008), the turtle body structure, for example the armoury, has remained remarkably conserved (Wyneken 1997).

All reptiles are amniotes, a major distinguishing feature separating this group from their amphibian predecessors, the first air-breathing tetrapods, which transitioned to land. The amniotic egg advanced the reptilian reproductive strategy, removing dependence on water

and switching to a terrestrial life cycle. Aquatic reptiles maintain this ancient connection to land for oviposition, with a few notable exceptions (e.g., viviparous sea snakes) (Jacobson 2007, Cogger 2014, Wellehan 2014). Sea turtles subsequently evolved from primitive land or aquatic chelonians approximately 110 million years ago (Salmon 2019). Despite re-adaptation to the marine environment, they remain bound to land for oviposition and to the atmosphere to breathe (Pritchard 1997, Witherington 2017).

Sea turtles are poikilothermic marine reptiles which fall into two families: Cheloniidae (also known as 'cheloniids', which include all species except the leatherback turtle; and Dermochelyidae (Witherington 2017). Externally, the bony shell of cheloniids is covered by scutes (epidermal-dermal unit), a thick outer keratinised epidermis supported by an underlying dermis (connective tissue, blood vessels, nerves), beneath which are dermal bones or osteoderms, centres of intramembranous ossification and shell growth (Boylan et al. 2017a). These dermal bones are an integral component of shell structure, as together with the ribs, they form the pleural bones, constituting the majority of the carapace (Figure 1.2) (Jacobson 2007, Mettee et al. 2017). Scute growth occurs as new layers of keratin are deposited along seams, the margins of which do not align with the underlying bones (Wyneken 2003). The keratin thickness of scutes varies between species, with the flatback turtle (*Natator depressus*) shell, often referred to as having a 'skin' covering. This is due to a thin stratum corneum layer and relatively low keratin content in the scutes (Boylan et al. 2017a). Having both soft external shell and skin, flatback turtles are particularly prone to trauma (evidenced by flipper tag tears and deep shell excoriations from mating). Another interesting observation is the frequent occurrence of growth of these scutes over barnacles (personal observation). The skin of the head, neck, and appendages is covered by (dermal) scales or thickened areas of epidermis (Rodriguez et al. 2018). Shedding of scutes and scales in freshwater turtles is thought to be continuous however further research in this area is required in sea turtles (Boylan et al. 2017a).

Leatherback turtles lack scales and scutes, and are instead covered by a leathery epidermis. In this species, rather than a fused bony shield, the carapace is made of a fibroadipose tissue (blubber) overlying the ribs and vertebrae, and a mosaic of ossicles (small polygonal

osteoderms) embedded in the skin, some enlarged to form longitudinal ridges (Wyneken 2001, Cogger 2014, Rodriguez et al. 2018). The leatherback skeleton retains substantial cartilage throughout life (Wyneken 2003).

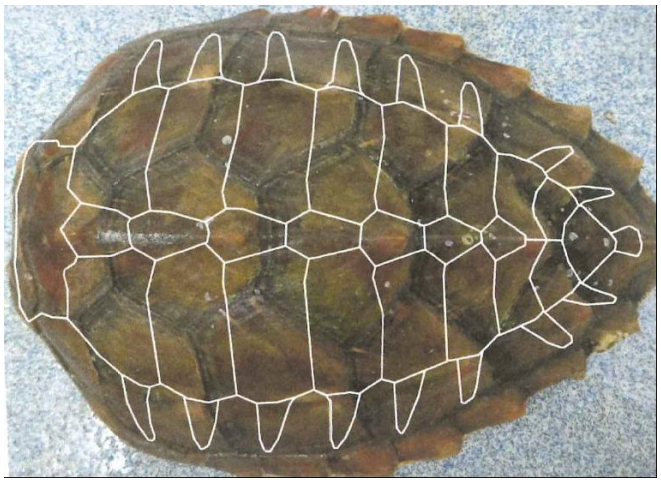

Figure 1.2 Image demonstrating scutes and carapacial bones are not aligned (Boylan et al. 2017a).

General modifications of the body and appendages include streamlined shape; rear flippers which function as rudders and are used with remarkable dexterity when nest-building; and elongated, flattened wing-like forelimbs for powerful propulsion (Pritchard 1997). Sea turtles literally 'fly' through the sea (Wyneken 2003), suiting their migratory lifestyle.

As a reptile, sea turtles have relatively few integumentary glands. The exception is the Rathke's gland, although its function is unknown (Figure 1.3). Hypotheses include immune function, conspecific communication (e.g., signalling for mass nesting during arribada), anti-fouling, and predator deterrence (Wyneken 2001, Boylan et al. 2017a). The pores are located in inframarginal scutes, prominent in ridley turtles (*Lepidochelys* spp.) and also in flatback turtles (personal observation). As a reptile, sea turtles do not possess lymph nodes and knowledge of sea turtle immunity is particularly limited (Zimmerman et al. 2010, Anderson et al. 2017).

As ectotherms, a functional immune system in sea turtles is temperature dependent, and comprises the spleen, thymus, pharyngeal- and gut-associated lymphoid tissue as well as bone marrow, including that existing within the shell (Howerth 2019). Thymic involution occurs with age, season, and disease state (Anderson et al. 2017). While increases in

immune cells such as melanomacrophages can occur with systemic inflammation, melanomacrophages and melanomacrophage centres can be found in the liver or spleen of sea turtles in good health, often increasing in number and size in older animals (Howerth 2019).

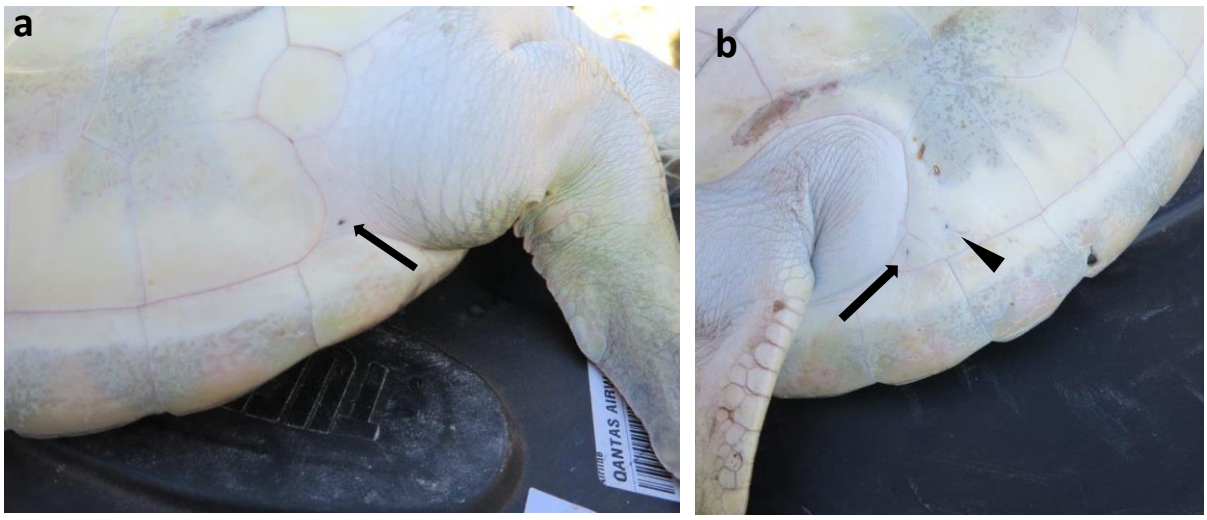

Figure 1.3 Juvenile flatback turtle with Rathke's pores visible on ventral surface from a cranial to caudal direction of (a) Most posterior-lateral axillary scale of the plastron (arrow), and (b) Caudal-most (fourth) inframarginal scale (arrowhead) and anterior-most inguinal scale (arrow) (photo credit Dr S. Whiting Department of Biodiversity, Conservation and Attractions, DBCA).

Sea turtles lack an external ear, the opening being covered by a tympanum (tympanic scale). Low frequency sounds are audible to sea turtles in water and air, but particularly underwater. Sea turtle olfactory sense organs function in water and air and are innervated by a comparatively large olfactory bulb. Vision is superior below water, which is useful for discerning predators and prey (Fritsches and Warrant 2013, Mader et al. 2017).

1.3.1 Diving adaptations

Sea turtles can reportedly spend over 90% of their time submerged without access to air (Williard 2013) because as air-breathing divers, their vital food and oxygen resources are separated by the water column. Subsequently they have developed many adaptations for diving and living a subaquatic life similar to marine mammals through convergent evolution (Lutcavage and Lutz 1997, Boylan et al. 2017b). Large diameter intrapulmonary airways reinforced with cartilage (up to respiratory bronchioles) and smooth muscle and elastin

(through all airways) (Solomon and Purton 1984), prevent airway collapse in cheloniids while facilitating high ventilation flow rates (Lutcavage and Lutz 1997). High tidal volumes allow for efficient and rapid gas exchange at the surface delivering nearly complete gas exchange in several breaths (Lutcavage and Lutz 1997, Boylan et al. 2017b). Similar to most reptiles, sea turtles have periodic breathing (a series of breaths followed by apnoea), anoxia tolerance (particularly high for the brain in sea turtles) and the ability to change metabolic rate (i.e., oxygen metabolism). Reptile haemoglobin has low oxygen affinity and can provide oxygen to tissues in a low oxygen environment (Boylan et al. 2017b). As with other reptiles, the stimulus for breath comes from a low blood oxygen concentration (Murray 2006).

Reptiles do not possess a diaphragm. Furthermore, the rigid shell of turtles prevents ventilation through costal movements (McArthur et al. 2004). Ventilation is therefore achieved via a change in intracoelomic pressure through movement of ventral abdominal muscles (*m. transversus* and *m. obliquus abdominus*), which compress the inguinal area and rotate the shoulder girdle (Wyneken 2001, Rodriguez et al. 2018). Both inspiration and expiration are active processes (Boylan et al. 2017b).

While there is no direct relationship between dive depth and duration in sea turtles (Lutcavage and Lutz 1997), deeper dives are usually longer and lower activity to reduce the rate of oxygen consumption. Leatherback turtles, that are capable of diving >1200 m have additional adaptations including a flexible cartilaginous carapace, pliant chest and collapsible lungs for the deepest dives (Fossette et al. 2010). As for duration, records show the longest period of breath-holding is seven hours, recorded in apparently overwintering loggerhead turtles (Hochscheid et al. 2005). This rejects the assumption that hibernation for several months occurs without breathing. With regards to the diving physiology of the flatback turtle, previous research indicated internesting flatbacks are shallow divers with very long dives (more than one hour) when compared to other species (Sperling et al. 2007). However, recent research on foraging flatback turtles in Roebuck Bay, WA has shown a mean dive depth of 10.8 m at foraging grounds with a shorter average dive duration (18.3 minutes) (Hounslow et al. 2022), compared to longer post-nesting migration dives of up to 210 minutes (J. Hounslow, Murdoch University, personal communication, August 2020).

The chelonian heart consists of sinus venosus, two atria, and a single ventricle, further subdivided into cavum venosum, cavum arteriosum, and cavum pulmonale, which are all interconnected (Rodriguez et al. 2018). Sea turtles have the ability to alter blood circulatory processes: shunting of pulmonary blood into the systemic circulation (or right to left cardiac shunt) occurs during apnoea associated with diving which adjusts blood oxygen levels, returning to systemic and pulmonary circulation (left to right shunt) with normal respiration (Garcia-Parraga et al. 2017). Sea turtles can also alter blood flow rate to conserve oxygen (lower heart rates when diving, versus higher heart rates when on the surface) (Williard 2013). The reptile heart lacks Purkinje fibres, and contractions are instead generated by myofibers (Rodriguez et al. 2018). This physiological response to diving apnoea with vagal bradycardia, increased pulmonary resistance, and reduced cardiac output resulting in right to left shunting, is commonly known as the dive reflex (Garcia-Parraga et al. 2017).

1.3.2 Water and electrolyte homeostasis

As with other reptilians, sea turtle kidneys function to maintain homeostasis through excretion and osmoregulation (Wyneken 2001). The kidneys lack distinct cortical and medullary areas, and the functional unit is the nephron (regenerated throughout life) (Solomon 1985). The kidneys lack a loop of Henle, and cannot concentrate their urine, excreting variable amounts of urea, uric acid, and ammonia (Wyneken 2001, Campbell 2012). Living in a salty, hyperosmolar environment, with the inability to excrete urine which is hyperosmotic to blood, has resulted in extrarenal salt excretion in sea turtles (Jacobson 2007), via modified and enlarged lacrimal or salt glands. These glands are located dorso-medially to the eye and not only function to excrete excess salt ions, but also to maintain homeostasis. These glandular excretions are often anthropomorphically misinterpreted by people as lacrimation. The oesophageal papillae also assist with salt regulation by expelling seawater during contraction of the oesophageal muscle, while retaining ingesta in the caudally-directed keratinised conical papillae (Lutz 1997, Rodriguez et al. 2018).

1.3.3 Aging and sexing

Defining age and sex is important for any demographic population survey. However, aging is challenging, as growth rates vary for individuals and biogeographically, resulting in adult maturation within a size range (Miller 1997). Generally, scute growth rings are unreliable for aging (McArthur et al. 2004), and while skeletochronology (counting rings in bone) is significantly more reliable, the major disadvantage is that bone samples must come from dead, stranded sea turtles (Avens and Snover 2013) as taking live individuals of an endangered species for age population structure research is unethical. Sexing is also challenging as sea turtles show little sexual dimorphism until adulthood. While diagnostic imaging, especially ultrasonography is a very useful technique, the resolution is insufficient to identify the sex of immature turtles (Innis et al. 2017b). Other studies have explored alternative methods for sex identification in turtles using blood and amniotic fluid for hormone assays (Gross et al. 1995, Xia et al. 2011, Tezak et al. 2020), with recent epigenetic work for sex determination in American alligators (*Alligator mississippiensis*) also very promising (Bock et al. 2022). However, these methods are not readily available and examination of the gonads by laparoscopy remains the most common method for sexing, as well as for assessing maturity and breeding status.

The gonads are paired organs. The male testes are flat, tan, and firmly attached to the kidney (Innis et al. 2017b). Maturity status can be determined by the shape and size of the testes and epididymides (Limpus and Limpus 2003). There is a single phallus for copulation, located in an elongated tail (sexually dimorphic feature) (Wyneken 2001), which is not involved in urination (Rodriguez et al. 2018). The female ovaries are cream, and have a granular appearance and incomplete attachment to the kidneys (Innis et al. 2017b), with maturity based on expansion of ovarian stroma and oviduct colour, shape and size, as well as degree of oviductal convolution (Limpus and Limpus 2003). When mature, vitellogenesis occurs about eight to 12 months prior to breeding (Innis et al. 2017b). Ovulated follicles enter the oviduct, where fertilisation occurs, then albumen and shell membranes are added, and the eggs are stored until deposition (Rodriguez et al. 2018). More mature follicles are located cranially, and immature follicles more posteriorly (Wyneken 2001). Recently

ovulated follicles leave a crater, ‘corpora luteum’ that regress into a scar, ‘corpora albicans’ (Wyneken 2001, Innis et al. 2017b), while un-ovulated vitellogenic follicles regress into ‘atretic follicles’. Observation of these reproductive features in females can provide an idea of previous reproductive activity (Limpus and Limpus 2003). Sea turtles are generally considered ‘capital breeders’ (Hamann et al. 2003, Perrault and Stacy 2018). This is based on the assumption they are inappetent during the breeding season and that all energy/fat reserves required to prepare for breeding have been accumulated prior to the season, which is thought to take more than a year (Miller and Limpus 2003). Sea turtles also display particular reproductive strategies including that they are long-living and slow to mature and have a low adult mortality (K-selected) along with high fertility and low survival of the early stages (r-selected features), which highlights the importance of survival of adults and large juveniles for population stability (Stacy et al. 2017a).

Even at necropsy, correctly identifying the sex can be challenging in immature animals. Histological assessment is typically required to accurately determine sex until individuals are at least >100 g (Innis et al. 2017b). Endoscopy has been used to determine sex and assess reproductive status (Divers et al. 2017). While these studies have been invaluable in understanding population structure, such procedures should involve the use of routine analgesia and sterilised equipment, in order to minimise adverse effects on the health and welfare of sea turtles (Divers et al. 2017).

1.3.4 Generalised sea turtle life cycle

The life of a sea turtle begins in the egg. Depending on sand temperature, incubation is complete six to nine weeks after oviposition, and hatching is facilitated by the embryo’s caruncle (egg-tooth). On average, it takes an additional three to five days for the hatchlings to emerge (usually between dusk and dawn), then orient to the horizon and enter the sea (also known as the ‘hatchling frenzy’ time period) (Lohmann et al. 1997). Hatchlings swim out into the open sea, drifting in open-ocean currents feeding on surface plankton during what is known as ‘The Lost Years’, aptly named as very little is known about this phase (Musick and Limpus 1997). All species have a pelagic, oceanic stage except flatback turtles, which appear to spend their entire lives in the neritic zone (on the continental shelf or less

than 200 m) and leatherback turtles, which appear to have a completely oceanic life (Walker and Parmenter 1990, Bolten 2003). Juvenile sea turtles arrive at coastal feeding grounds after around five to 10 'lost' years, where they remain until sexual maturity (15-50 years), at which point, sexually mature male and female turtles migrate back to their natal beach (up to several thousand kilometres away). Their life cycle is sometimes described as a 'series of migrations' (Lohmann and Lohmann 1998).

Until recently, the longest migration ever recorded was by a leatherback turtle travelling from its nesting beach in Indonesia 20,885 km to foraging grounds off Oregon in the north-west of the United States of America (USA) (Lohmann et al. 1997, Dutton 2007). The new record for longest migration is 40,011 km by a loggerhead named 'Yoshi' who was released in 2017 after 20 years in captivity at Two Ocean Aquarium, South Africa. Her last satellite transmission was off the coast of Western Australia (WA), near Eighty Mile Beach (October 2020) which is expected to be in her home range (Two Oceans Aquarium 2020). While not completely understood, hatchling imprinting, geomagnetism, and chemoreception have been implicated in (aiding) this natal homing phenomenon (Lohmann et al. 2013).

Most courtship occurs in mating grounds close to the nesting beach (Miller 1997, Musick and Limpus 1997). Females are receptive about two weeks prior to ovulation of the first clutch (Hamann et al. 2003). Mating can be brutal with females sustaining bites to neck, flippers, and head from males and damage to the shell by the males' claws that can take lengthy periods to heal (and develop secondary infections). Males also attack and bite the trailing flippers and dorsal tail of other copulating males when competing for a female mate (see Section 1.5.5 – Trauma). Males and females both mate with multiple partners and females can store sperm from several males, resulting in mixed paternity clutches (Miller 1997). Further genetic evidence supports males also opportunistically mating with females along migratory paths (FitzSimmons et al. 1997).

Females come to shore for oviposition, running through an innate process comprising six stages: emerging (from water), body pitting (digging a pit for the body using fore-flippers), egg chambering (excavating nest for eggs with rear flippers), egg laying, covering up (infilling egg chamber and camouflaging nest), and returning (to water). Each season, the female will

lay three to five clutches of approximately 50-150 pliable eggs every two weeks (inter-nesting period) (Department of Parks and Wildlife 2013b). The whole process can take approximately one to several hours, which for various reasons, can sometimes result in an unsuccessful nesting, for reasons such as unsuitable conditions, disturbance (more likely during the early stages of nesting), and returning to the sea without egg-laying. Parental care is limited to the female selecting a suitable nesting site (Department of Parks and Wildlife 2013b). At the end of the season, sea turtles return to their foraging grounds, migrating back for breeding every one to nine years (remigrant interval) depending on individual, environmental, and species factors (Miller 1997, Hamann et al. 2003).

Like most reptiles, sea turtles display a distinct breeding season (Denardo 2006, Innis et al. 2017b), which is related to a temperature change, and for sea turtles it is generally in the warmer months (summer nesters). However, there are exceptions to the rule with some year-round nesting recorded in the most northern parts of the state (e.g., olive ridley turtles, *Lepidochelys olivacea*) and a large winter nesting flatback rookery at Cape Domett in the Kimberley region of WA (Commonwealth of Australia 2017b).

1.3.5 Diet and habitat

Sea turtles display uneven geographical distribution to exploit different habitats based on their dietary preference and temperature tolerances (Miller 1997). Their highly diverse niche specialisation, facilitates coexisting in the same environment (Lovich et al. 2018).

Generally, young sea turtles (post-hatchlings and juveniles), drift in the open-ocean, feeding on zooplankton (except the flatback turtle mentioned below), and while some species are known to feed in association with rafts of *sargassum* (a floating foraging ground rich in plant and small animals) (Spotila 2004), little is known about the early life stages of flatback turtles, including feeding behaviour and diet. Once sea turtles (except for the leatherback turtle) undergo the ontogenetic shift into their nearshore foraging grounds, diet specialisation commences. The most significant ontogenetic dietary shift is for the green turtle (*Chelonia mydas*) which transforms from a carnivorous to predominantly herbivorous

diet (Arthur et al. 2008a). While not an exhaustive list, general known habitat and dietary items for the various sea turtle species are shown below in Table 1.1.

Table 1.1 Generalised diet and habitat of adult turtles of the six species that frequent coastal WA.

Species	Diet	Habitat
Green turtle	Seagrass, algae, mangrove fruit. Primarily herbivorous but still eat some invertebrates; jellyfish and fish carrion.	Shallow, nearshore embayments with abundant seagrass beds; algae-rich coral and rocky reefs; estuaries.
Loggerhead turtle	Benthic invertebrates mainly shellfish (crustaceans, molluscs, echinoderms); jellyfish and occasionally fish.	Hard and soft-bottomed habitats, sandy reefs, coral reefs, shallow bays, seagrass meadows.
Flatback turtle	Soft-bodied invertebrates (sea pen, soft corals, sea cucumbers) and including jellyfish as well as cuttlefish.	Soft-bottomed, shallow, turbid inshore waters.
Hawksbill turtle	Sponges (primary diet), shellfish, algae, seagrass, soft-bodied invertebrates (including jellyfish).	Coral reef, other hard-bottoms, seagrass pastures.
Olive ridley turtle	Shellfish, soft-bodied invertebrates (including jellyfish), algae.	Soft-bottomed, shallow coastal or oceanic foraging areas.
Leatherback turtle	Jellyfish specialist and other gelatinous creatures in water column (e.g., pelagic sea squirts).	Completely pelagic, oceanic.

(Bjorndal 1997, Miller 1997, Limpus 2007, 2008a, Heithaus 2013, Commonwealth of Australia 2017b, Witherington 2017, Department of the Environment and Energy 2019b, f, d, e, c, a)

1.3.6 Temperature-dependent distribution

Sea turtle distribution is influenced by ocean temperature (Hamann et al. 2013), with turtles normally inhabiting waters above 15-20°C isotherms, leading to a concentration around the tropics decreasing with increasing latitude (Wallace et al. 2010a, Wallace et al. 2011). The

exception to this is the leatherback turtle, the only partially endothermic reptile, found in waters between 0-12°C, observed actively seeking out temperate foraging grounds (Spotila et al. 1997, Bolten 2003, Innis and Staggs 2017). The other species have varying tolerance to cold temperature and as ectotherms have a minimum temperature threshold below which normal body functioning ceases (e.g., 8-12°C; see Section 1.5.7 – Cold-stunning) (Figure 1.4). While most sea turtles spend the majority of their time in subtropical and tropical waters (particularly hawksbill, flatback, and olive ridley turtles), green and loggerhead turtles (*Caretta caretta*) also utilise temperate habitats (Commonwealth of Australia 2017b, Witherington 2017) (Figure 1.5 and Figure 1.6). Sea turtles exhibit behavioural thermoregulation, for example, flatback turtles have been observed basking at the surface for hours (Bustard 1972). However, for all species, sand temperatures between 25-35°C are required for successful incubation. Therefore nesting occurs in tropical areas including northern parts of Australia (Ackerman 1997, Cogger 2014, Department of the Environment and Energy 2019b) (Figure 1.6). Similarly to other reptiles, sea turtles show temperature-dependent sex determination (TSD), with a pivotal temperature (approximately 27-30°C) producing a sex ratio of 50:50, and above this temperature producing more females, and below, more males (Hamann et al. 2013).

Figure 1.4 Green turtle under Kwinana grain terminal jetty, Perth (32°S).

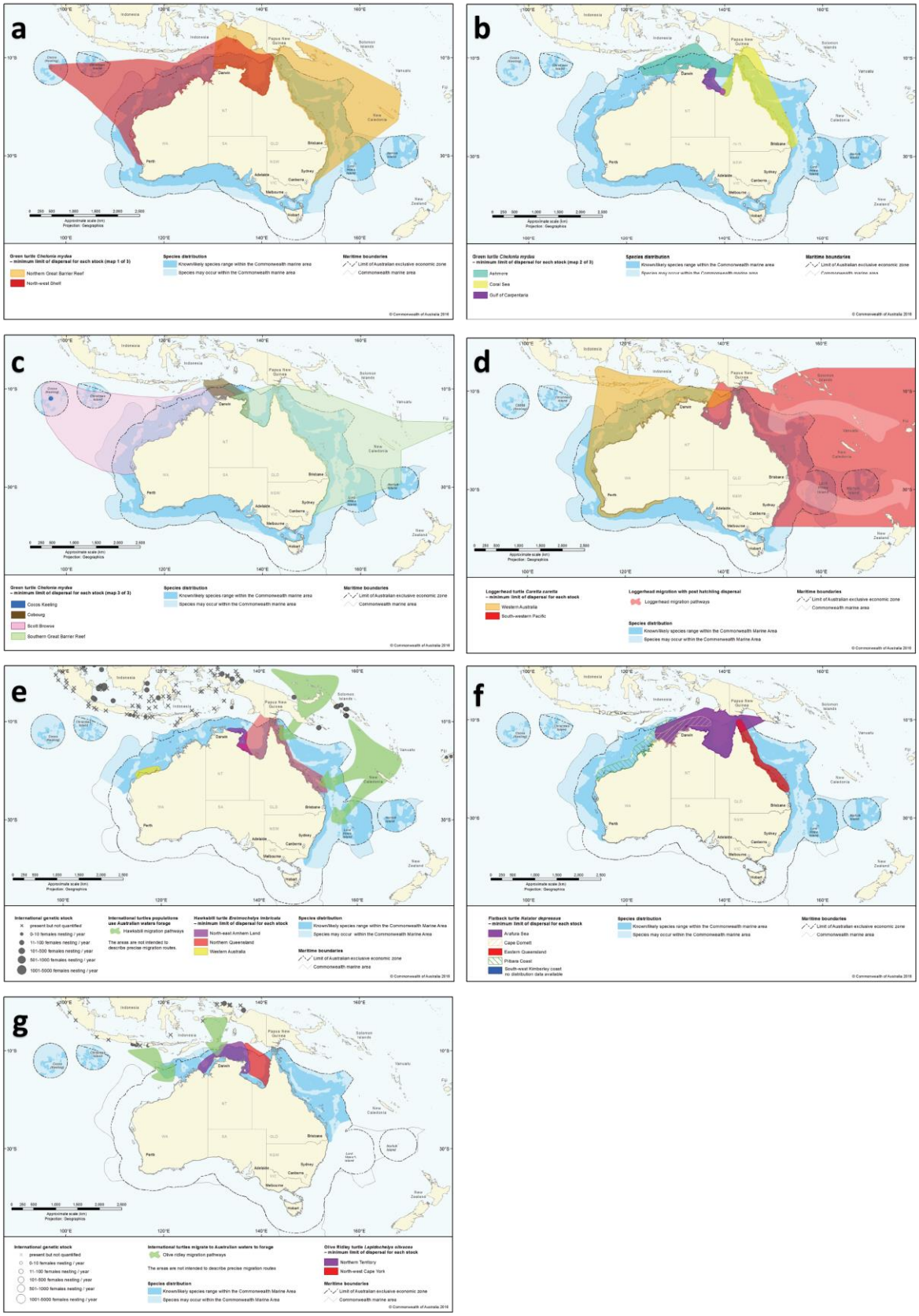

Figure 1.5 Distribution maps of foraging sea turtle dispersal for (a–c) Green, (d) Loggerhead, (e) Hawksbill, (f) Flatback, and (g) Olive ridley turtles (Commonwealth of Australia 2017b).

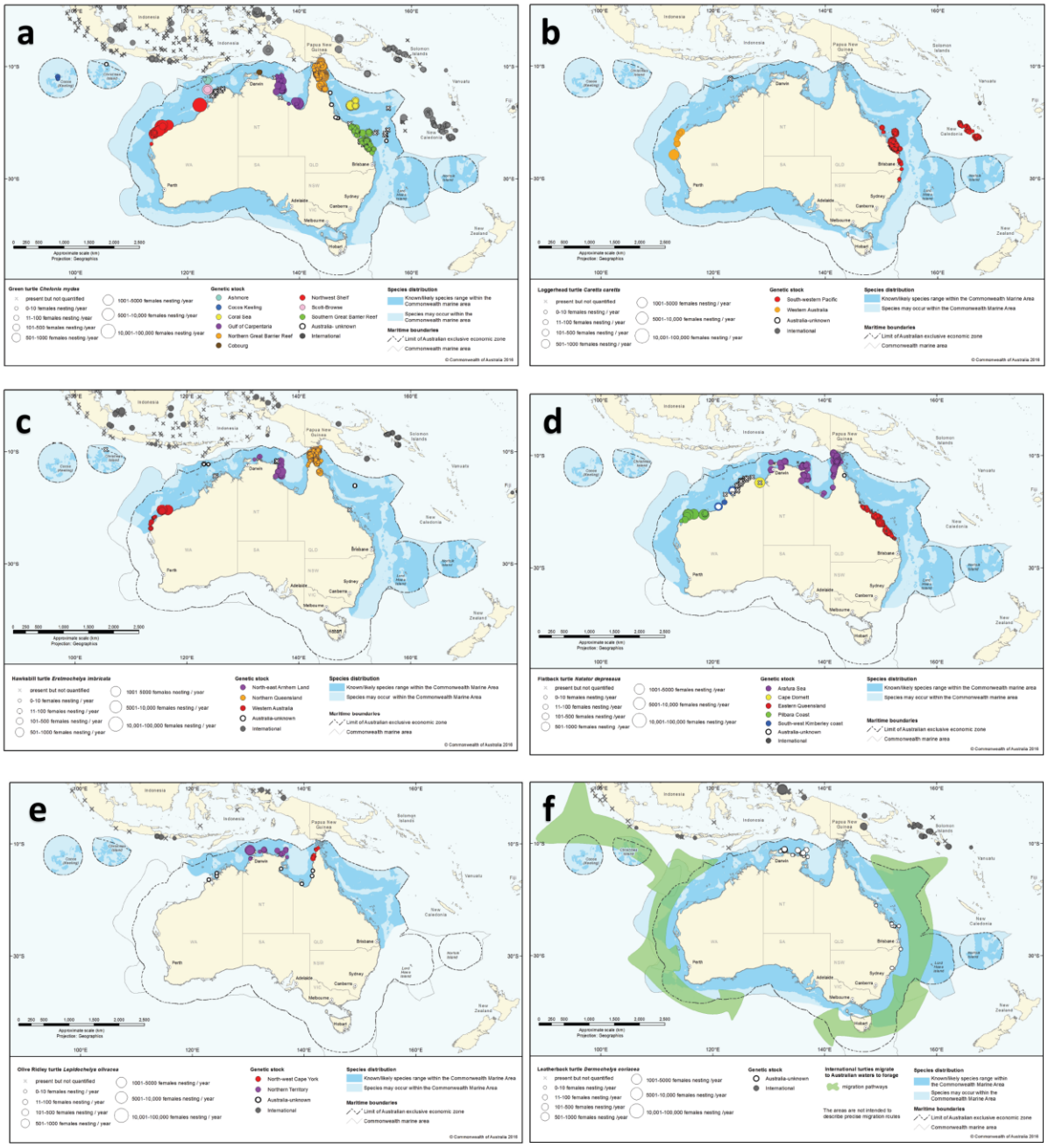

Figure 1.6 Distribution maps of nesting stocks of sea turtles in Australia for (a) Green, (b) Loggerhead, (c) Hawksbill, (d) Flatback, (e) Olive ridley, and (f) Leatherback nesting and foraging dispersal map (Commonwealth of Australia 2017b).

1.4 Conservation status and threatening factors

All six species found in WA are classified as threatened fauna and protected under Federal (*Environmental Protection and Biodiversity Conservation Act 1999*) and State (*Biodiversity Conservation Act 2016*) legislation. Globally, Australia is obligated to protect sea turtles under international agreements including *Convention of the International Trade in Endangered Species of Wild Fauna and Flora* (CITES), *Convention on the Conservation of Migratory Species of Wild Animals* (CMS) and the *Indian Ocean and South East Asian Marine Turtle Memorandum of Understanding* (IOSEA Marine Turtle MoU) (Commonwealth of Australia 2017b).

At both state and Commonwealth levels, the flatback, green and hawksbill turtle (*Eretmochelys imbricata*) are listed as 'vulnerable' and the loggerhead and olive ridley turtle are listed as 'endangered', whereas the leatherback turtle is listed as 'vulnerable' and 'endangered' status under state and federal legislation respectively. Internationally, the World Conservation Union (IUCN) Red List further classifies the loggerhead, leatherback and olive ridley turtles as 'vulnerable', the green turtle as 'endangered', the hawksbill turtle as 'critically endangered', while the flatback turtle, remains classified as 'data deficient' (IUCN 2019). Owing to its restricted range, the flatback turtle is particularly vulnerable to catastrophic environmental events and Australia has both international and national obligations to improve the understanding of this endemic data deficient species (Pritchard 1997).

Despite the IUCN being the most widely recognised authority for global assessment of conservation status, the Red List's capacity to adequately assess sea turtle status (i.e., risk of extinction) is hotly debated, as it is for other wide-spread, long-lived migratory species such sharks and cetaceans (Wallace et al. 2011). Subsequently, the concept of regional management units (RMUs), which comprise biologically and geographically defined subpopulations of sea turtles in different ocean basins, has evolved to address the inadequacies of the global system (Wallace et al. 2010a). A total of 58 RMUs have been identified internationally, of which the leatherback and loggerhead RMUs have been

adopted by IUCN (Rees et al. 2016). This has led to differing conservation status categories corresponding to various threats and risks across regions, for example, subpopulations of northern Indian Ocean loggerhead turtles are 'critically endangered', while those in the southern Indian Ocean are 'near threatened' (Casale and Tucker 2017). Western Australian stocks all belong to south-eastern Indian Ocean RMUs, of which there are five (Wallace et al. 2010a). In Australia, sea turtle populations are further divided into smaller biological units via genetic analyses. Twenty two distinct stocks, each with its own evolutionary history have been identified and Australia's obligation to conserve genetic biodiversity is recognised in the Recovery Plan for Marine Turtles in Australia (Commonwealth of Australia 2017b).

1.4.1 Threats

Sea turtles face a multitude of threats, which impact at all stages of their life cycle. The significance of each threat varies between populations and regions (Lutcavage et al. 1997, Bolten 2003, Epperly 2003, Milton and Lutz 2003, Wallace et al. 2010a). There are numerous ways of grouping the various threats, such as: natural vs anthropogenic (direct and indirect); life stage affected. Below, the major threats are grouped into functional categories, which align with the Recovery Plan for Marine Turtles in Australia (Table 1.2), with WA specific examples where possible. Not unexpectedly, the vast majority of the threats are anthropogenic (Lutcavage et al. 1997, Heppell et al. 2003, Aguirre and Lutz 2004, Commonwealth of Australia 2017b, Witherington 2017) (see Section 1.2 – Health and disease investigations for further explanation of health impacts). However, while strong evidence exists that anthropogenic factors are having the greatest impact on sea turtle survivorship, increasing human activities in the near-shore zone shared with sea turtles has the potential to skew the impact of human activities on turtles (Flint et al. 2015a).

Table 1.2 Risk-threat matrix for prioritising Australian stock conservation status indicating disease and pathogen threats remain predominantly unknown (Commonwealth of Australia 2017b).

Species-Stock:	G-sGBR	G-CS	G-nGBR	G-GoC	G-Cobourg	G-NWS	G-AR	G-ScBr	G-CK	LH- swPac	LH-WA	F-eQld	F-ArS	F-CD	F- swKim	F-Pil	H-nQld	H-neArn	H-WA	O- nwCY	O- NT	LB nesting
Stock Status	↗	?	↙	?	?	→	?	?	↗	↙	→	→	?	?	?	?	↓	?	?	↓	?	↓
THREAT																						
A. Climate change and variability																						
B. Marine debris – entanglement		U						U			U											
B. Marine debris – ingestion						U					U		U						U	U	U	
C. Chemical and terrestrial discharge – acute																						
C. Chemical and terrestrial discharge – chronic																						U
D. International take – outside Australia's jurisdiction						U		U														
D. International take – within Australia's jurisdiction						U		U														
E. Terrestrial predation					U		U	U													U	U
F. Fisheries bycatch – international												U	U									
F. Fisheries bycatch – domestic																						
G. Light pollution																						
H. Habitat modification – infrastructure/coastal development			*																			
H. Habitat modification – dredging/trawling																						
I. Indigenous take					U																	
J. Vessel disturbance																						
K. Noise interference – acute						U						U					U					U
K. Noise interference – chronic	U			U		U		U		U	U	U		U	U	U	U	U	U	U	U	U
L. Recreational activities					U																	
M. Diseases and pathogens	U	U	U	U	U		U	U			U	U	U	U	U	U	U	U	U	U	U	U

Stock Status: ? = unknown, → = stable, ↗ = recovering, ↘ = early stages of decline, ↓ = declining
Risk rating: pink = very high, yellow = high, blue = moderate, green = low, U = unknown
* Historical guano mining

Key for stocks listed in Table 8

G-sGBR = green turtle southern Great Barrier Reef
G-CS = green turtle Coral Sea
G-nGBR = green turtle northern Great Barrier Reef
G-GoC = green turtle Gulf of Carpentaria
G-Cobourg = green turtle Cobourg
G-NWS = green turtle North West Shelf
G-AR = green turtle Ashmore Reef
G-ScBr = green turtle Scott Reef – Browse Island
G-CK = green turtle Cocos Keeling
LH-swPac = loggerhead turtle south-west Pacific
LH-WA = loggerhead turtle Western Australia
F-eQld = flatback turtle eastern Queensland
F-ArS = flatback turtle Arafura Sea
F-CD = flatback turtle Cape Domett
F-swKim = flatback turtle south-west Kimberley
F-Pil = flatback turtle Pilbara
H-nQld = hawksbill turtle north Queensland
H-neArn = hawksbill turtle north-east Arnhem Land
H-WA = hawksbill turtle Western Australia
O-nwCY = olive ridley turtle north-western Cape York
O-NT = olive ridley turtle Northern Territory
LB = leatherback turtle

Habitat modification and coastal development

Habitat modification and coastal development includes infrastructure such as ports, marinas, oil and gas developments, coastal urbanisation and aquaculture (Commonwealth of Australia 2017b). Development results in loss, degradation or fragmentation of nesting and foraging habitat, affecting population viability through reduced egg recruitment, subclinical and clinical disease (e.g., through disturbance, malnutrition, injuries and general deterioration in environmental health) (Lutcavage et al. 1997, Heppell et al. 2003, Commonwealth of Australia 2017b). While WA's coastline is relatively free from urban development, certain regions including the Pilbara and offshore areas (North-West Shelf, NWS) are highly industrialised. In spite of this, literature on impacts of industry on sea turtles in WA are scarce (Whittock 2017). While not specific to WA, large-scale industry can be devastating to sea turtles due to the sheer size of these operations and the wide range of impacts including light disorientation on land or in water, increased commercial shipping traffic, dredging, marine constructions and associated turbidity, explosions, industrial noise, and major beach erosion (Dobbs 2001). Habitat protection is key to conserving any wildlife species. In WA, many flatback rookeries and foraging grounds are adjacent to large-scale resource development projects, highlighting the importance and urgency of further research into this species.

Pollution: Marine debris

Marine debris includes both ingestion of and entanglement in anthropogenic debris, including rubbish (e.g., plastic bags, plastic bottles, packaging), and discarded fishing gear (e.g., nets, synthetic ropes, fishing line). The majority of the debris is plastic and mostly from land-based sources and not unexpectedly, plastic is the most commonly ingested pollutant (Schuyler et al. 2014, Nelms et al. 2016a). Plastic does not biodegrade like natural products, instead it disintegrates into tiny pieces or microplastics (<5 mm) which can persist in the environment for decades to centuries (Thevenon 2014). This debris adsorbs toxins and can leach other toxins like plasticisers into the environment or the animal if ingested. These microplastics make their way into the food chain, concentrating in the larger, long-lived predators, which is of course a major concern to humans (Thevenon 2014). The debris can

provide a platform for colonisation of marine organisms such as algal growth which in turn attracts the algae-specialist green turtle, and can also harbour pathogens or act as a vector of alien species posing a potential biosecurity risk (Thevenon 2014). Marine debris has emerged as a global epidemic degrading the health of oceans and marine life, especially sea turtles (Commonwealth of Australia 2017b). The indiscriminate eating habits of sea turtles places them at risk, especially hatchlings and juveniles inhabiting convergence zones where floating debris concentrates. Ingestion can result in numerous sequelae, ranging from the sub-lethal effects of poor nutritional intake, to toxicoses and potentially fatal gastrointestinal (GI) blockage (Bjorndal 1997) (see Section 1.5.3 – Buoyancy disorders). As we continue to use plastic for single use items and short-lived consumer products, the volume of plastic polluting the environment continues to grow.

Ocean and shore-based clean-ups serve an important role in marine debris management, providing valuable categorical data and reducing litter in the ocean such as nurdles (small plastic pellets about the size of a lentil, the building blocks of almost all plastic products) which spilt from a South African ship in 2017 and started washing up on the south-west coast of WA (Gubana 2018). However, this is a global issue, which needs to be urgently addressed at the source (Wilcox et al. 2016, Commonwealth of Australia 2017b). Despite accounting for a small proportion of total marine debris, discarded fishing gear (nets, ropes, monofilament lines, trawls, and gill nets) cause the greatest number of sea turtle entanglements (Commonwealth of Australia 2018). These ‘ghost nets’ (or ‘ghost gear’) are made from synthetic plastic fibres (e.g., nylon) manufactured to resist degradation, which drift around the ocean indiscriminately killing wildlife for decades (Thevenon 2014). While there is only the occasional report from WA (Prince 2007), northern Australia has some of the highest reported rates of entanglement in the world (Wilcox et al. 2014). Entanglements can reduce mobility, inflict physical injuries (e.g., lacerations and amputations) and potentially result in death (e.g., infection or drowning) (Lutcavage et al. 1997). Fishing gear from recreational sources is also a significant contributor to harmful marine debris (Commonwealth of Australia 2018).

Pollution: Noise

Noise pollution includes acute or chronic noise from domestic, commercial or industrial sources. While little information is available in sea turtles, the adverse impact of noise is likely to be similar to other species, and more extensive at industrial levels. Negative impacts include physical, physiological and behavioural derangements, and displacement or disturbance of normal activities (Lutcavage and Lutz 1997, Mader et al. 2017). Our oceans are becoming noisier, and the global growth of oil and gas industries adds to this increasing noise. The threat posed by noise pollution associated with oil and gas industries is particularly relevant to WA. The current Recovery Plan for Marine Turtles in Australia recognises noise as a threat and recommends further research, government regulation and mitigation (Nelms et al. 2016b, Commonwealth of Australia 2017b).

Pollution: Artificial light

Artificial light pollution sources include lights on jetties, vessels, platforms, and industrial and urban lighting. Disorientation from artificial lighting can disturb nesting sea turtles but mainly affects hatchlings when attempting to reach the lowest brightest light (the horizon) and occurs on land or in-water, resulting in exhaustion or predation (Pendoley and Kamrowski 2015, Wilson et al. 2018). While mitigation guidelines restricting light usage have been developed to minimise nesting sea turtle disturbance through the 'Turtle Watchers' Code of Conduct' (Department of Parks and Wildlife 2013b), regulations surrounding artificial lights in industry, despite the far greater scale, are significantly more challenging to manage (due to use of lighting for safety issues and the presence of international vessels in Australian waters) with WA sea turtles identified at high risk (Western Australia Environmental Protection Authority 2010, Kamrowski et al. 2012).

Pollution: Chemical and terrestrial discharge

Chemical and terrestrial discharge pollution includes toxic and non-toxic pollutants from urban, agricultural, and industrial run-off, effluent, or spills. These contaminants can be chemical in nature (e.g., pesticides, herbicides, and oils), heavy metals, agricultural compounds (e.g., fertiliser), or sediment. While precise disease expression mechanisms are

yet to be elucidated, there are links between chemical contaminants and disease (e.g., fibropapillomas are more common in polluted areas), while non-toxic pollutants can cause subclinical disease (e.g., through food depletion, decreased water quality) and indirectly cause disease outbreaks from biotoxic algal blooms associated with eutrophication (Lutcavage et al. 1997, Dobbs 2001). The recent red tide in Florida which began in October 2017 and lasted over a year caused the greatest number of sea turtle deaths in a single event on record, with a death toll of 589 (Chow 2019). While no sea turtles have been reported as affected by biotoxins in WA, *Lyngyba* blooms are a fairly regular occurrence in the waters adjacent to the Broome townsite (Roebuck Bay) and their occurrence is likely to increase with climate change (Arthur et al. 2008b, Estrella 2013) (see Section 1.5.6 – Toxicoses for more details). This is of great significance considering Roebuck Bay has been identified an important habitat foraging green and flatback turtles of mixed genetic stocks (Dr S. Whiting, Department of Biodiversity, Conservation and Attractions, personal communication, 19 February 2019).

Climate change and other extreme weather events

Threats to sea turtles as a result of climate change are alarming. It is predicted the impacts on the reproductive phase will be the worst, as there are clear relationships between an increase in sea temperature, sea levels, and storm activity on the success of nesting, hatching, and incubation (Hamann et al. 2013) such as nest inundation, and population feminisation due to TSD (Janzen 1994). In addition to the many indirect effects of climate change such as ocean acidification, increased atmospheric carbon dioxide, and global marine species redistribution, climate change has been implicated in contributing to emerging diseases, with disease predicted to further increase with climate change (Harvell et al. 2002, Hamann et al. 2013, IPCC 2014). Additionally, extreme weather events are anticipated to increase in frequency and intensity with climate change. However only a few studies have investigated the relationship of environmental conditions to sea turtle or other marine wildlife strandings (Limpus and Reed 1985b, Limpus et al. 2012). Flint et al. (2017) demonstrated delayed sea turtle strandings, seven to 12 months following flooding events in Queensland, with the lag time shortened for those in embayments (bays with higher

discharge concentration). While Preen et al. (1995) showed the main driver for mortalities and population declines of dugong (*Dugong dugon*) in 1992 at Hervey Bay, Queensland, was a combination of the flood-cyclone-flood and the associated seagrass loss. Similar events have occurred in WA, with marine heat waves implicated in green turtle and dugong chronic wasting syndromes and mortalities associated with seagrass dieback in Shark Bay (Thomson et al. 2015). Recently, an ulcerative dermatitis known as 'freshwater skin disease', has emerged in dolphins worldwide, including in the Swan-Canning river system in Perth, WA and is associated with environmental factors such as hyposalinity (Duignan et al. 2020). During this project, we reported a mass mortality event in north-west WA near Broome involving finfishes, sea turtles and sea snakes, associated with *Streptococcus iniae*, a potentially emerging bacterial disease of marine reptiles, where extreme weather events including a marine heat wave were also implicated as potential stressors (see Chapter five) (Young et al. 2020).

Feral animals and other predators

Hatchling sea turtles face very high rates of predation with estimates that only one in 1000 to 10,000 survive to adulthood (National Oceanic and Atmospheric Administration 2022). Examples of terrestrial predators include natural (e.g., goannas, seagulls, dingoes, birds of prey, crabs) and introduced species (e.g., foxes, cats, dogs, pigs), which predate on eggs and/or hatchlings on the beach (King 2016, Commonwealth of Australia 2017b). Aquatic predators include large predatory fish, octopus, crocodiles, and sea lions (Heithaus et al. 2008, Whiting and Whiting 2011, Wilson et al. 2019). Sea turtles are one of the major prey items of sharks, especially tiger sharks (Heithaus et al. 2008). However, sea turtles can survive major shark attacks (Heithaus et al. 2008) with a recent study in the flatback turtle proposing sea turtles may have adapted anti-predator aggressive behaviour in response to the inability to retreat into their shell (Hounslow et al. 2021). The large size of the flatback hatchling is another proposed adaptation to reduce predation on land and in the water (considering their entire life is spent on the continental shelf, contending with more predators than open waters) (Bolten 2003). Introduced predators or natural 'opportunistic'

predators (e.g., seagulls) also thrive in disturbed areas, further increasing levels of predation.

Interaction with fisheries

Sea turtles have the highest bycatch intensity of marine megafauna (Lewison et al. 2014). Some species are attracted to the nets by the presence of prey items or have learned scavenging behaviours (Heithaus 2013, Parga et al. 2017). This can significantly impact populations given that adults, with higher reproductive value, are predominantly affected (Wallace et al. 2010b). Prince (2007) reported leatherback turtles being caught on long-lines and crab pots offshore in WA. In general, sea turtles that died as a result of fisheries interactions were healthy beforehand, similar to vessel strike victims (Barco et al. 2016). Interaction with fisheries can secondarily end in forced submersion, resulting in death by drowning. Individuals that survive are subsequently at increased risk of predation, and potentially decompression sickness (DCS) also known as 'the bends', which was recently discovered in sea turtles, where gas bubbles accumulate in the blood vessels and tissues which may result in fatal gas embolism (Lutcavage and Lutz 1997, Garcia-Parraga et al. 2017). However, to date DCS has not been identified in WA sea turtles. Since compulsory introduction of Turtle Exclusion Devices (TEDs) in WA and the rest of Australia, the risk of capture of turtles as bycatch in trawl nets has been significantly reduced (State of Queensland 2018). Interestingly, evidence suggests flatback turtles have superior respiratory capabilities compared to other sea turtles, with the ability to survive forced submersion in prawn trawling nets longer than other species (Sperling et al. 2007). This may reduce mortality rates from accidental bycatch (Sperling et al. 2007).

Vessel strike

Vessel strike is a clear example of a direct anthropogenic threat, occurring more commonly in populated areas with increased recreational or commercial vessel traffic. Vessel strike can inflict severe injuries and is the threat most likely to result in death (Barco et al. 2016). As dead stranded sea turtles frequently present in an advanced state of decomposition, it is often difficult to determine the health status of the animal prior to the vessel strike (i.e., an

unhealthy turtle may have been at increased risk of strike due to an existing debilitation such as GI impaction or entanglement) or whether the vessel strike occurred pre or post-mortem (although this can usually be determined with histological examination) (Epperly et al. 1996, Chaloupka et al. 2008, Barco et al. 2016).

Miscellaneous

Other threats specific to WA include nuclear testing at the Montebello islands which would have caused countless mortalities from the initial thermal and shock impacts (Limpus 2002, Johansen et al. 2020), with additional health-related impacts of radioactive exposure to subsequent generations and population declines yet to be reported.

Diseases and pathogens

Diseases were highlighted as an area of major knowledge deficit both in Australia and globally (Hamann et al. 2010, Commonwealth of Australia 2017b, Mashkour et al. 2020), and can be infectious and non-infectious in nature, and often the result of imbalance of the host-pathogen-environment relationship (see Section 1.5.8 – Infectious diseases for further discussion).

1.4.2 Risk factors for threatening processes

Globally, climate change, fisheries bycatch, and coastal development are ranked highest in a global threat prioritisation risk assessment for sea turtles (Donlan et al. 2010, Wallace et al. 2011). Climate change equally ranks highest in Australia, but this was not the case for fisheries interactions. However, the opposite is true for the Mediterranean region (Lucchetti et al. 2016). Entanglement ranks second highest, but is primarily a regional problem affecting sea turtles in the Top End (northern Australia), including the Gulf and Arafura Sea (Commonwealth of Australia 2017b, State of Queensland 2018). Fuelled by the ongoing 'tortoise-shell' trade in our neighbouring countries, hawksbill turtles are the most at-risk group for international take, and this is the only threat considered 'very high' risk in WA. While some threats are global and widespread, for example, climate change, these examples of regional and species-specific threats support the need for management of RMUs

accordingly and highlight the importance of international agreements and cooperation in conserving migratory species.

As previously mentioned, hatchlings are the most at-risk life stage, with the survival rate and reproductive value increasing with age (Musick and Limpus 1997, National Research Council (NRC) 2010, Bolten et al. 2011). Strategic conservation management is crucial in these long-lived, slow maturing animals, and threats should be scaled according to reproductive value i.e., focused on adults and subadults. Some argue that even removal of low numbers from these groups is unsustainable (Heppell et al. 2003, Stacy et al. 2017a), and that providing further support through rehabilitation of even a single individual is worthwhile. By the same token, customary take for traditional use requires careful management within indigenous communities, to ensure harvests are sustainable for long-term population viability (Bjorndal and Jackson 2003, Wallace et al. 2008).

1.4.3 Limitations and management of threats

There are a great deal of challenges when studying sea turtles. Aside from occasional basking sea turtles, only the nesting females ever return to land, and for first-time nesters this may not have been since hatching themselves some 20-50 years prior (Limpus 2008a, b, Cogger 2014). Additionally, sea turtles spend their life at sea and most of the time submerged (Lutcavage and Lutz 1997, Williard 2013). The majority of data available focus on the terrestrial component of the life cycle, with rather large knowledge gaps regarding the aquatic stages of the life cycle. Due to the vastness of the marine environment and potential unknowns surrounding contributing factors, quantifying indirect anthropogenic threats is particularly difficult (Harvell et al. 2002, Herbst and Jacobson 2003). While direct impacts may be more easily quantifiable, the number affected can only be considered a minimum index due to reasons including, but not limited to, at-sea deaths and unreported and unrecovered strandings from remote unpatrolled areas (Epperly et al. 1996, Dobbs 2001, Flint et al. 2015a).

Most population estimates are derived from nest abundance, mark re-capture studies, and some demographic studies from in-water laparoscopy. While there is a trend of population

decline globally (Seminoff and Shanker 2008), some populations appear to be recovering (Balazs and Chaloupka 2004, Wallace et al. 2011), albeit slowly due to their long generation times (Domiciano et al. 2017). In Australia, the southern Great Barrier Reef and Cocos Keeling green turtle stocks are both recovering (Commonwealth of Australia 2017b). Recovery may be achieved by removing the threat (commercial harvest for the Great Barrier Reef stock) or maximising reproductive output (protecting nesting beaches for Cocos Keeling stock). Although complicated due to the issue of shifting baselines (populations first recorded after noting declines renders baselines misrepresentative) (Bjorndal and Jackson 2003), these trends provide evidence that mitigation and recovery efforts are succeeding (Jackson et al. 2001, Balazs and Chaloupka 2004, Chaloupka et al. 2008). Historically, direct anthropogenic factors have played a significant role in population decline. In WA, sea turtles were harvested for shell, meat, and eggs until the commercial cannery closed in 1973 (Limpus 2002). While it is debatable as to whether the commercial industry in the state was indeed small-scale (Halkyard 2014), large sea turtle populations remain in WA. Other effective management strategies, including 'go slow zones', TEDs, and marine protection areas, have reduced some of these direct anthropogenic impacts, or at least in areas in which implementation and enforcement occurs (Flint et al. 2015a).

As previously explained, all threats to sea turtles have the potential to affect health either directly (e.g., GI blockage through marine debris ingestion) or indirectly (e.g., loss of body condition through coastal development and habitat degradation) and possibly lead to disease. The way threatening processes result in disease, however, is complex and often not well understood, especially for natural and anthropogenic environmental threats. Anthropogenic threats, while causing the most serious impacts to turtle health, can often be managed (Aguirre and Lutz 2004, Flint et al. 2010d, Flint et al. 2015b, Witherington 2017). Without baseline health and disease information combined with a multidisciplinary approach to understand how these threats impact the survival of sea turtles, management strategies will continue with varying levels of success (Harvell et al. 1999, Flint 2013). The following section discusses the two most common infectious diseases dominating investigations in the field of sea turtle disease and health (spirorchiidiasis and

fibropapillomatosis) and provides brief descriptions of other common diseases and syndromes.

1.5 Common diseases and syndromes

1.5.1 Spirorchiidiasis

Spirorchiidiasis is a disease exclusive to marine and freshwater turtles caused by spirorchiid trematode parasites (subclass: Digenea; family: Spirorchiidae) (Stacy 2008). As inhabitants of the circulatory system, these parasites are frequently referred to as 'blood flukes' and cause damage through the pathology associated with the adults and the eggs (Werneck et al. 2016). Establishing spirorchiidiasis as a definitive cause of death should be made with caution because the presence of spirorchiids and associated pathology can be an incidental finding, and the primary diagnosis/cause of death is often unrelated (Stacy et al. 2010a). Further, while spirorchiids are more common in debilitated sea turtles, even physically robust individuals may have a heavy spirorchiid trematode burden with severe accompanying lesions. This suggests spirorchiids not only cause disease opportunistically, but can act as primary pathogens, and that disease is likely a continuum where effects of cumulative factors for example nutrition and immunity, reach a threshold, above which disease ensues (Stacy et al. 2010a). Despite the uncertainty, spirorchiids are still recognised as a significant contributing cause of morbidity and mortality in sea turtles and ongoing research is being undertaken to determine the significance of these important parasites to both individuals and populations (George 1997, Flint et al. 2010d, Greiner 2013).

Spirorchiidiasis is one of the oldest recorded diseases in sea turtles with reported prevalence as high as 100% in some studies (Dailey et al. 1991, Stacy 2008), however little is known of the life history of these parasites. This is not surprising considering the vastness and complexity of the marine environment (Glazebrook et al. 1989).

There is considerable taxonomic debate in classifying spirorchiids. To date 29 species of marine spirorchiids, representing 10 genera, have been identified as parasites of sea turtles (Platt and Blair 1998, Stacy et al. 2010a, Werneck et al. 2016, Chapman et al. 2019). Adult

spirorchiids are morphologically diverse ranging from linguiform to filiform; classically separated on morphological criteria, for example the presence (distomous) or absence (monostomous) of a ventral sucker (Glazebrook et al. 1989, Stacy 2008). Spirorchiid eggs come in a range of shapes and sizes which fall into three broad categories based on morphology, for example type I eggs have hooked terminal processes (Wolke et al. 1982, Gordon et al. 1998a, Flint et al. 2010a).

Preliminary research in this field (Work et al. 2005, Stacy 2008) suggests many spirorchiid species are found in a variety of sea turtle host species ('generalists'), while others appear to be host-specific and are only found in one species ('specialists') (Santoro and Mattiucci 2009). However, systemic monitoring in understudied geographic areas and host species improves our understanding of this disease including distribution of spirorchiid species (Santoro et al. 2020). For example, the marine spirorchiid, *Carettacola hawaiiensis*, was considered to be host-specific to *C. mydas* of Hawaii (Work et al. 2005, Stacy 2008), until this species was detected in *C. mydas* in a previously understudied geographic location (Western Pacific, Australia) (Chapman 2016, Chapman et al. 2017). Of the sea turtle species found in Australia, spirorchiids have only been reported in *C. mydas, C. caretta, E. imbricata*, and recently in *L. olivacea* (in Brazil), but have not yet been reported in *D. coriacea* or *N. depressus* (until this study) (Stacy 2008, Jerdy et al. 2016). Flint et al. (2009b) also reported higher frequencies of spirorchiid infestation in Australian waters than in other regions.

Until recently, the life cycle was unknown for marine spirorchiids; therefore, it had to be inferred from freshwater species, which have two hosts, the intermediate host, a gastropod (pulmonate for the freshwater life cycle), and the definitive host, a turtle (Pinto et al. 2015) (Figure 1.7). Miracidia, the free-living motile stage of the spirorchiid, are released from eggs on contact with water, which then enter the snail. These miracidia become sporocysts in the intermediate host which then develop into fork-tailed cercariae (larval stage), and after emergence, penetrate through the skin or mucosal membranes of the definitive host i.e., not through ingestion (Stacy 2008, Pinto et al. 2015). These cercariae develop into adults, which produce eggs. Both the adults and the eggs circulate through the cardiovascular system as emboli, causing other significant pathology in various organs. Following this the

turtle sheds the eggs and the life cycle continues (Stacy 2008). Central miracidium have also been observed in eggs shed from sea turtles (Gordon et al. 1998a, Stacy et al. 2010a). While there are still many unknowns, three studies have recently identified the intermediate stage of sea turtle spirorchiids in marine invertebrates (including a limpet, worm snail, and polychaete worm) for the first time using molecular techniques, a step towards better understanding the marine spirorchiid life cycle (Stacy et al. 2010b, Cribb et al. 2017, de Buron et al. 2018).

Spirorchiid eggs can affect most host organs, and tend to cause more damage than adult parasites (Werneck et al. 2016). Eggs are deposited in the blood stream by adults which either remain where oviposition occurred (primary site) or disseminate throughout the body and embolise various organs (secondary site). These eggs which become lodged in small vessels incite an inflammatory response, potentially leading to impaired function and even death (Chapman et al. 2016b, Werneck et al. 2016).

Clinical signs can be related to the organ system affected by the spirorchiids however, clinical signs are not always associated with pathology or even severity of infection. This general lack of correlation between clinical signs and pathology is also reported in other reptile species (Flint et al. 2010d) (Figure 1.8).

While spirorchiids can be found in all organs, some spirorchiids appear to have organ-specificity, the frequency of which varies between studies, as does the associated pathology (Gordon et al. 1998a, Stacy 2008). For example, *Neospirorchis* spp. are considered to be the most clinically significant spirorchiids, with tissue tropism for the brain and central nervous system. These species have been implicated in mass mortality events (Jacobson 2006). Different studies report variable results, with *Hapalotrema* spp. usually reported in the heart and major arterial vessels (Glazebrook et al. 1989, Gordon et al. 1998a, Stacy et al. 2010a). However, this may be biased as these sites are readily accessible for examination as opposed to smaller vessels and the central nervous system (Stacy 2008). Gastrointestinal spirorchiidiasis is also relatively common, with interference of myenteric ganglia function in the cases of severe spirorchiid inflammatory lesions resulting in GI motility disorders, leading to impactions (Flint et al. 2009b).

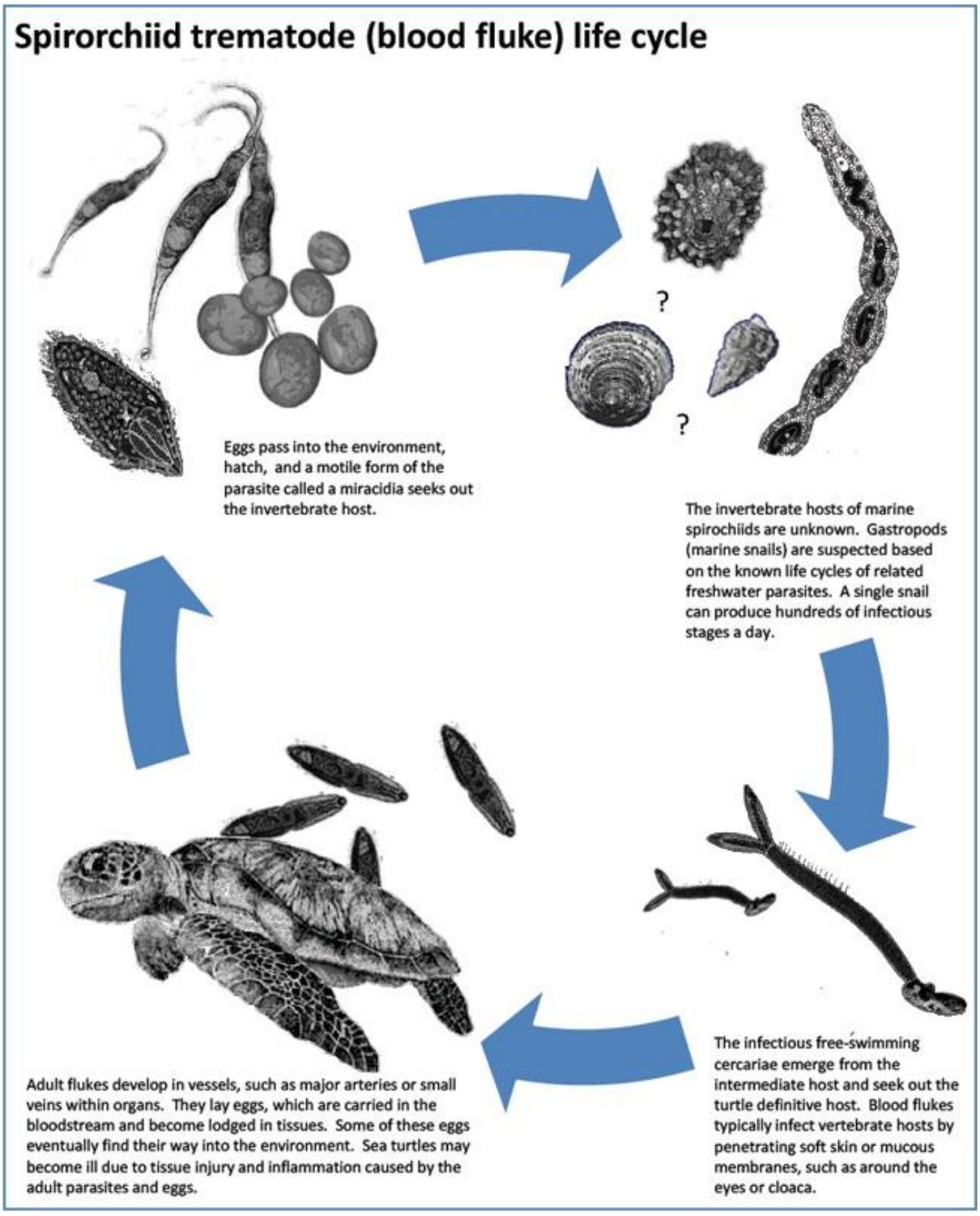

Figure 1.7 Putative life cycle for sea turtle spirorchiids (Mettee 2014).

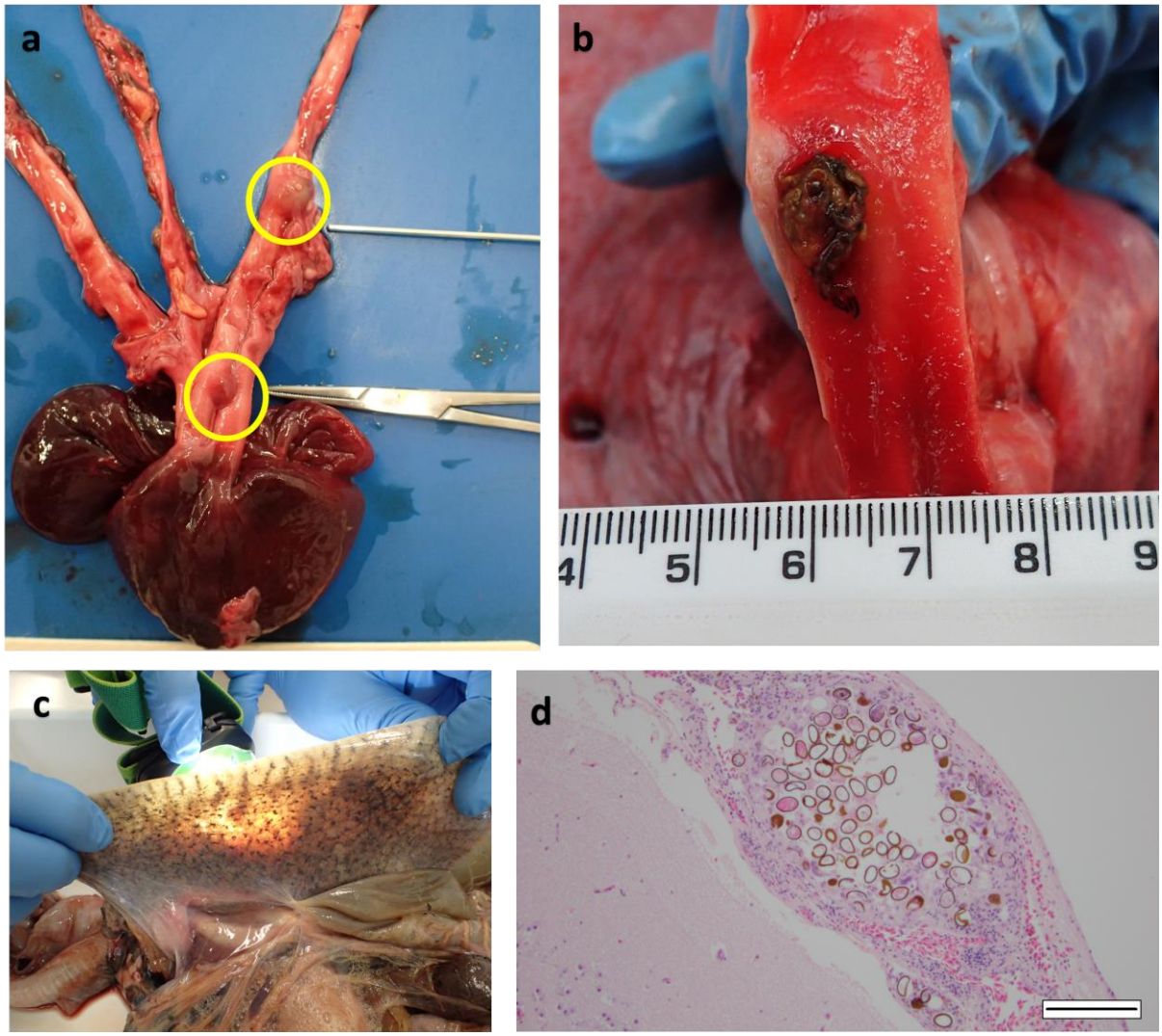

Figure 1.8 (a) Cardiovascular spirorchiidiasis with multiple left aortic aneurysms, (b) Inside aneurysm showing thrombus caused by adult spirorchiids, (c) Severe pulmonary spirorchiidiasis caused by egg granulomas, and (d) Histomicrograph of neurological spirorchiidiasis showing egg granuloma in meningeal vessel (H&E, x40, scale bar 500 µm).

While detection techniques are constantly improving, Flint et al. (2010d) also suggest the pathogenicity of spirorchiids is increasing over time due to diagnosis of spirorchiidiasis increasing in prevalence between studies.

As no spirorchiids have been found in previous mortality studies of pelagic sea turtles (Oros et al. 2016), it has been suggested sea turtles become infected on entering near-shore foraging habitats (Work et al. 2005). Although green turtles undergo an ontogenetic shift on entering the near-shore environment, changing from a mostly carnivorous to predominantly herbivorous diet (Aguirre et al. 1998), spirorchiid infection likely occurs from inadvertently

ingesting the gastropod intermediate host found in seagrass beds (Stacy 2008). Other risk factors for spirorchiid infection include warm weather and immaturity. Flint et al. (2010d) hypothesised that increased GI spirorchiidiasis in warmer seasons is due to increased cercarial emergence or marine gastropod availability, or that warmer temperatures stimulate egg production. While higher egg burdens are predicted to occur in younger animals because of reduced host immunity when spirorchiid exposure is increased in nearshore foraging grounds, in general, animals in better condition had lower egg counts possibly due to decreased fecundity of parasites in these host types (Work et al. 2005).

Classical parasitological methods involve gross necropsy, low magnification light microscopy, and morphological identification of spirorchiids. To diagnose disease, Gordon et al. (1998a) suggests careful gross examination is as effective as histopathology and much less expensive. While Jacobson (2006) proposes histopathological results and spirorchiid presence do not necessarily implicate spirorchiids as the cause of morbidity and mortality, but as a contributor to a multifactorial disease process.

The diagnosis of the intensity of infection is also challenging, as the number of adult parasites doesn't correlate with the lesion severity (Stacy et al. 2010a). In addition, quantification is difficult due to the small size and intravascular location (Gordon et al. 1998a). Even when this disease was first observed, a lack of correlation between the number of eggs and adult flukes was noted (Stacy 2008). Other challenges include difficulty accessing fresh carcasses and sea turtles stranding late in the course of the disease, often with multiple disease processes occurring concurrently, which further complicates disease diagnosis (Gordon et al. 1998a).

Detection of spirorchiids in intermediate hosts is difficult due to the diversity of potential hosts in sea turtle habitats coupled with low prevalence and wide dispersion (Stacy et al. 2010b). Monitoring trematodes for cercariae emergence and dissection of gastropods using classical techniques is a monumental task in terms of the numbers of gastropods which would require screening and although feasible, such an exercise would be extremely time consuming (Stacy 2008).

Recent increased spirorchiid molecular characterisation research, suggests it is a valuable technique and may overcome many limitations of the classical detection methods (Chapman et al. 2016b). Molecular methods can be used to identify species, which can then be correlated with species-specific pathology and can be used to assess parasite status, prevalence in a population or response to treatment (Flint 2013). Additional benefits include the potential for ante-mortem diagnosis through the development of indirect ELISA tests for detecting spirorchiid antibodies (Work et al. 2005).

Molecular methods can also be used to rapidly screen large numbers of marine gastropods with greater sensitivity and specificity. Molecular techniques are able to detect one egg in 1.5 g of tissue (equivalent to the earliest prepatent infection) (Stacy et al. 2010b). Stacy et al. (2010b) also documented the first evidence of the intermediate stage of a marine spirorchiid, *Learedius learedi,* in an intermediate host, the keyhole limpet, *Fissurella nodosa,* using molecular methods. Over 4000 gastropods were tested and only one positive PCR result was obtained in *F. nodosa*, with repeated testing yielding no further positives. In addition, molecular work is particularly useful for ova, as identification to species is limited by the lack of differentiating characteristics (Chapman et al. 2016b).

It is likely molecular technology will continue to grow in the parasitology field and contribute to our knowledge about this poorly understood disease. At present, a combination of morphological, histological, and molecular methods, is likely the best approach for accurate identification of the marine spirorchiid or a diagnosis of spirorchiidiasis.

1.5.2 Fibropapillomatosis

Fibropapillomatosis (FP) is a common, neoplastic disease of sea turtles with worldwide distribution (Aguirre and Lutz 2004, Work et al. 2004). Fibropapillomatosis was first reported in *C. mydas* at the New York Aquarium in 1936 (Smith and Coates 1938). Following this, the disease received very little attention until the 1980s (Herbst 1994), when the incidence and distribution of the disease significantly increased. Numerous epidemics have recently been reported, and FP has reached panzootic status in *C. mydas* (Jones et al. 2016). Despite initial reports dating back over 70 years, many aspects of this disease including the primary

aetiological agent remain unknown. However, research efforts are underway to understand and manage this important disease of sea turtles (Aguirre and Balazs 2000).

Disease prevalence ranges from 0% to 92% in studied populations, with significant variation observed between nearby localities (Balazs 1991, Limpus and Miller 1994). For example, a 50% prevalence of FP was present in the Indian River *C. mydas* population in Florida, while <1 km away, FP was not observed in the Sabellariid worm reef population (Herbst 1994, Limpus and Miller 1994). This variation in prevalence, coupled with the epizootic nature of the disease, suggests FP is an infectious disease (Herbst 1994). The successful transmission of this disease experimentally between individuals, further suggests the involvement of a viral agent (Herbst et al. 1995). Chelonid Herpesvirus 5 (ChHV5), an alphaherpesvirus of the genus scutavirus, has been implicated as the most likely aetiological agent. However, the inability to isolate and culture the virus in vitro, means Koch's postulates cannot be fulfilled, hence the causative agent whilst suspected cannot be confirmed (Herbst 1994).

The lack of reporting of FP prior to the 1980s, suggests FP is still an emerging disease. Prior evidence of this disease would be unlikely to go unnoticed due to the pathognomonic nature of the disease, which often has a severe and obvious clinical presentation (Jones et al. 2016). This condition is visually characterised by external tumours, comprising single or multiple, smooth or wart-like, sessile or pedunculated, raised cutaneous masses, which range from 0.1 cm to more than 30 cm in diameter (Herbst 1994). Tumours can also affect all internal organs but these visceral tumours are less common and more difficult to detect. Visceral tumours can be identified with diagnostic imaging (e.g., CT, MRI, radiology, ultrasonography) and laparoscopy in live turtles. However, as most visceral tumours are discovered during necropsy, the detection data for these tumour types are subsequently skewed (Jones et al. 2016) and the prevalence is likely to be underestimated. Aguirre and Lutz (2004) estimate approximately 25-30% of sea turtles with external tumours, also have internal tumours. While a 10-year study in Hawaii did not find any turtles with internal tumours that did not also have external tumours (Work et al. 2004). It has been suggested that internal tumours develop during the later stages of disease progression and their presence is a predictor of poor survivability (Herbst et al. 1999, Jones et al. 2016).

While the disease is far more common in *C. mydas*, it has been described in all sea turtle species (Harshbarger 1991, Aguirre et al. 1999, D'Amato and Moraes-Neto 2000, Huerta et al. 2002). However, FP is yet to be histologically confirmed in *N. depressus* (Limpus and Miller 1994). While FP was first observed in Australia during the 1970s, official records were not published until 1996 (Raidal and Prince 1996). In Australia and other specific locations, for example Puerto Rico, despite the seemingly severe disease, FP does not appear to be a significant cause of mortality (Flint et al. 2010d, Patrício et al. 2012). This is in direct contrast to other studies, where FP is implicated as the primary cause of stranding (Hirama and Ehrhart 2007). Although fibropapilloma tumours are histologically benign, this disease can potentially cause severe debilitation due to space-occupying effects or interference with systemic function (Flint et al. 2009b). Despite these conflicting findings, it has been generally concluded that FP does not appear to significantly impact sea turtle survivability, however, developing further understanding of this disease has been highlighted in a multidisciplinary, multinational report focused on global research priority areas including threats such as disease (Hamann et al. 2010). Additionally, two multinational reports on global research priorities (Hamann et al. 2010, Wallace et al. 2011) both conclude effects of pathogens are data deficient. A substantial amount of work has since been undertaken in this area including a recent health baseline study demonstrating virus enzooticity with no differences between health variables for chelonian herpesvirus positive and negative cases (Page-Karjian et al. 2020). Another study found altered immune system function due to reduced natural killer cell activity in turtles with FP compared to tumour-free turtles (Perrault et al. 2021).

Juvenile sea turtles are most commonly affected by FP, and tumour development typically occurs after recruitment to the neritic habitat. Fibropapillomatosis has not been detected in pelagic post-hatchlings or in new recruits (Herbst 1994). Aguirre et al. (1998) found a strong correlation between straight carapace length (SCL) and FP, where sea turtles in Kaneohe Bay with ≤40 cm SCL were free of FP and ≥45 cm SCL had FP. Potential reasons for new recruits being more at risk of contracting FP include migration, new environment, increased population density, change in diet and exposure to the pathogen (Jones et al. 2016). A major concern in relation to the impact of this disease on juveniles, is that aside from adults, larger

juveniles have a higher reproductive value than other demographic classes (for example hatchling or small juveniles) and changes to survival of this size-class may have significantly negative impacts on the population (Herbst 1994).

Larger sea turtles also tend to have larger tumours suggesting FP is a chronic disease as it is presumed tumours which develop later in life would be smaller than those that develop earlier (Work et al. 2004). Tumour scores have been developed to quantify the severity of disease based on size and number of external tumours which, can in turn be used as a predictor of survivability (Balazs 1991). In live sea turtles, those with higher tumour scores show pathology results indicative of immunosuppression, chronic inflammation, and systemic bacterial infections (Aguirre et al. 1995, Work and Balazs 1999).

Fibropapillomatosis frequently manifests as primary growths in the periocular tissue, spreading to other epithelial areas (Aguirre and Lutz 2004) (Figure 1.9). This clinical presentation is likely to reflect sea turtle behaviour and disease transmission (Work et al. 2004). Transmission is likely to be horizontal as sea turtles tend to develop disease on entering the neritic waters, co-inhabited by the marine leech, *Ozobranchus margoi,* and the saddleback cleaner wrasse, *Thalassoma duperrey*. Both of these species have been implicated as the potential vectors for the disease (Flint 2013, Jones et al. 2016).

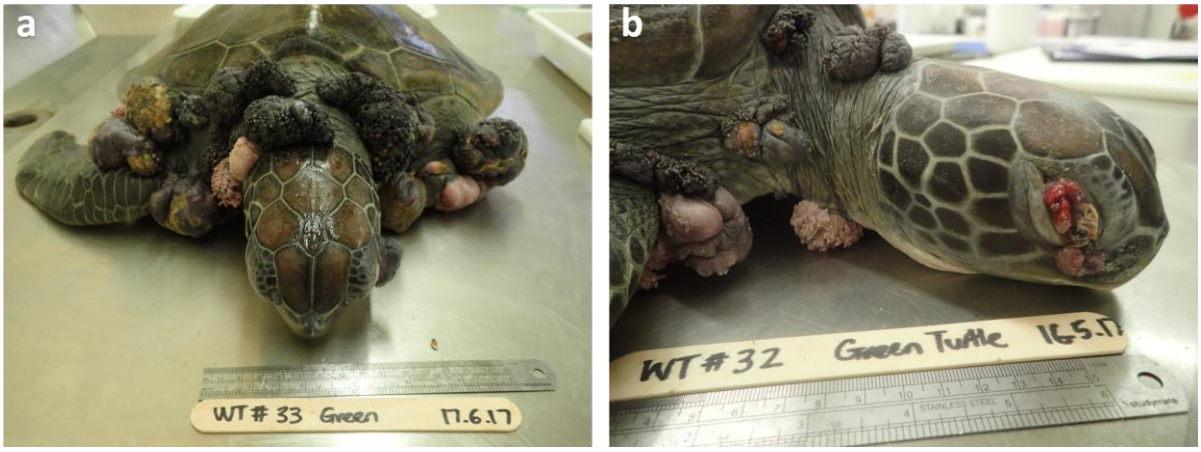

Figure 1.9 Typical presentation of green turtles with severe fibropapillomatosis (a) Rostro-caudal view, and (b) Lateral view showing periocular and ocular FP obscuring vision.

It has been suggested there is a developmental progression through the various stages of disease from papilloma (early) through fibropapilloma (intermediate), fibroma (chronic), and to fibrosarcomas of low-grade malignancy (Herbst 1994, Work et al. 2004). Common histological findings include epidermal hyperplasia, acanthosis, orthokeratosis, basal cell degeneration, spinous layer degeneration, and dermal-epidermal cleft formation (Herbst et al. 1999). While no direct association has been established, spirorchiid eggs are frequently found in FP tumours (Flint et al. 2009b).

Chelonid herpesvirus 5 has been implicated as a potential cause of FP, due to its strong association with the disease (Boylan et al. 2017a). It is unknown whether single or multiple agents are involved, but it is generally accepted as having a multifactorial aetiology (Aguirre and Lutz 2004). Environmental factors have also been strongly associated with the expression of this disease. Many researchers have shown a strong correlation between FP prevalence in areas adjacent to agriculture, industry, and urban development (Aguirre et al. 1994, Herbst 1994, Adnyana et al. 1997b, George 1997). However, this trend was reversed in Puerto Rico with FP prevalence higher at a pristine site when compared to another local area with increased human activity (Page-Karjian et al. 2012). Attempts to identify a relationship between FP rates and increased eutrophication due to anthropogenic activities have been unsuccessful to date due to chemicals and others pollutants being excluded from the water quality assessments (dos Santos et al. 2010, Jones et al. 2016). Water temperature also appears to play a role in the development of FP lesions. Growth is promoted during the warmer summer months, which then develop into debilitating lesions by autumn, resulting in an increased rate of stranded sea turtles with FP in the winter months (Herbst 1994).

While sea turtles are resilient to physical damage, they appear to be sensitive to biological and chemical insults (Aguirre and Lutz 2004). The presence of chemicals in the environment is hypothesised to be part of the multifactorial problem leading to FP with certain chemicals potentially disrupting the neuroendocrine system or acting as immune toxins or carcinogens (Herbst 1994, Jones et al. 2016). Work et al. (2014) challenged biotoxicological investigations attempting to link eutrophication and algal blooms with FP rates. Instead Work et al. (2014) aimed to highlight the inciting cause of the bloom, or the cause of the FP, not the bloom

itself. While only a small number of environmental contaminants have been tested, low levels when compared with other species, and lack of correlation between chemicals (e.g., POPs), trace metals (e.g., selenium), and other pollutants (e.g., organophosphates), suggest their potential role in expression of FP is likely to be minimal (Aguirre et al. 1994, Keller et al. 2014). However, the involvement of other contaminants may be revealed in future investigations (Jones et al. 2016).

Molecular evidence has reported that ChHV5 is not novel and has co-evolved with sea turtles over many years. While no direct links have been made, this strongly suggests recent emergence of FP is related to anthropogenic-related environmental change (Jones et al. 2016). Other advances in diagnostics include the improved sensitivity and specificity of testing for FP whereby ChHV5 has now been amplified from tissues collected from sea turtles with and without clinical disease (Page-Karjian et al. 2012). Additional molecular work has shown a strong geographical role in the transmission of the virus due to different variants in the various geographical areas and that the same variants are found in sympatric sea turtle species (Herbst 1994).

Despite heightened public awareness, strong evidence suggests FP is an emerging disease globally due to the pathognomonic nature of this disease (Jones et al. 2016). Further research should target knowledge gaps including identification of the aetiological agent, transmission routes, and contributing environmental factors, with molecular investigations likely to have a strong influence on the direction of future research. As previously mentioned, sea turtles may serve as sentinels of marine environmental health due to the longevity, site fidelity, and other aspects of the life history, as such using prevalence and severity of FP as an environmental indicator has important implications for humans as co-inhabitants of this shared marine environment.

1.5.3 Buoyancy disorders

One of the most common causes for stranding of sea turtles is related to floating, a syndrome caused by a variety of factors which can involve a number of organ systems (Manire et al. 2017b). One of the most common reasons for floating is ileus (gastrointestinal

stasis) usually caused by dehydration, systemic disease, and impactions (by natural food items or marine debris). In the case of impactions, gas builds up cranial to the obstruction leading to floating (Manire et al. 2017b). These animals have difficulty diving to feed in the water column or on the benthos, further perpetuating the starvation and dehydration cycles. Obstructions can also lead to life-threatening perforation and septicaemia. Other potential causes of floating include anaerobic gas-forming bacteria, penetrating injury (e.g., propeller strike), lung disorders, coelomic gas, neurological dysfunction, and inanition (Wyneken et al. 2006). Cold water can also lead to floating (Innis and Staggs 2017). Marine debris ingestion is highly concerning and while many pieces may pass uneventfully (observed in successfully rehabilitated and released sea turtles), only one piece may be required to cause death (Bjorndal et al. 1994, Manire et al. 2017b, Wilcox et al. 2018). The sub-lethal effects include displacement of nutritious food by debris, malabsorption, malnutrition, inanition, and absorption of toxins and plasticisers (Bjorndal 1997); all of which can affect systemic health and potentially cause floating (see Pollution: Marine debris in Section 1.4.1 – Threats). While the underlying cause of floating needs to be addressed, considering the role of dehydration in gastrointestinal ileus and floating, identifying and correcting hydration is a key factor when treating this disease (Manire et al. 2017b).

1.5.4 Chronic debilitation

Chronically debilitated sea turtles are a common end point of malnutrition, and account for a large proportion of rehabilitation patients (Manire et al. 2017c). While the inciting cause is infrequently known, potential causes include starvation, malnutrition (e.g., due to impaction), GI disorders (e.g., bacterial or parasitic gastroenteritis), cold water, trauma, pneumonia, and other diseases such as spirorchiids. These sea turtles present with chronic signs of emaciation (e.g., dehydration, cachectic myopathy, plastron concavity, carapace softening), lethargy, and heavy epibiota (Stacy et al. 2018b). These turtles are in a state of catabolism often with opportunistic pathogenic infections and intense spirorchiidiasis (Stacy et al. 2017c). It appears that chronic diseases sustained over winter can result in clinical disease in the warmer months, evidenced by increased strandings at the end of winter and early spring (Manire et al. 2017c). This trend was also observed in WA with increased

strandings in mid-late winter and early spring. Rehabilitation is typically protracted as the animals present at the end stage of disease (Manire et al. 2017c).

1.5.5 Trauma

Physical injury commonly results from anthropogenic-related activities including vessel strike, interaction with commercial/industrial machinery, and entanglement, but can also result from natural causes such as shark attack and conspecific mating wounds (Mettee and Norton 2017). Anthropogenic trauma mainly affects the carapace and skull due to the occurrence of collisions generally at the surface. For example, a vessel strike can cause parallel slicing from the propeller or cavitation from the skeg or vessel hull, resulting in lacerations and fractures (Mettee et al. 2017) breaching the protective barrier which facilitates secondary opportunistic infections (see Section 1.5.8 – Infectious diseases below). Open shell fractures can also result in buoyancy issues (see above). While sea turtles can survive severe physical affliction such as amputations, of all the causes of mortality, trauma from vessel strike is most likely to end in death (Orós et al. 2005, Chaloupka et al. 2008, Work et al. 2015, Barco et al. 2016).

1.5.6 Toxicoses

While increased toxins and heavy metals have been associated with nervous, endocrine, neoplastic, and immune disorders in marine wildlife including sea turtles, the pathogenesis of toxicities including the cumulative and synergistic effects of such contaminants are not well understood (Kannan et al. 2000, Holyoake et al. 2011, Poppenga 2017, Barraza et al. 2021). Although sea turtles can withstand significant physical injuries they are particularly intolerant to chemical agents and biotoxins (i.e., cyanobacteria and microalgal toxins) (Lutcavage et al. 1997). Sea turtles exist in the air-water interface, impacted by environmental toxins both through consumption or the respiratory (inhalation) route for example inspiration of volatile gases (e.g., biotoxin aerosols and oil spill vapours) when surface breathing. Chemicals such as oil can also cause sloughing and necrosis of tissue (George 1997). Their lack of avoidance behaviour, indiscriminate feeding, and site fidelity makes them particularly susceptible to the cumulative chronic effects of prolonged exposure

to pollutants (Milton and Lutz 2003). Of additional concern are microplastics and toxicities associated with ingestion (see Section 1.5.3 – Buoyancy Disorders, and, Pollution: Marine debris in Section 1.4.1 – Threats) (Nelms et al. 2016a, Manire et al. 2017a, Poppenga 2017). Additionally, levels of heavy metal and regularly measured toxins are an order of magnitude lower in sea turtles than other marine species (Milton and Lutz 2003). A study from NWS in WA found flatback turtles had even lower levels of Hg than other sea turtle species (Schneider et al. 2022). Recent research highlights the need for more baseline screening and *in vitro* studies, as a non-lethal way to investigate the toxic effects on these threatened species (Finlayson et al. 2016, Villa et al. 2017).

1.5.7 Cold-stunning

Sea turtles cannot function when exposed to temperatures below 8-10°C, becoming lethargic, anorexic, positively buoyant, and moribund (Milton and Lutz 2003). Cold-stunned animals are previously healthy before ending up in cold waters due to a sudden drop in water temperature within their normal seasonal range. Alternatively, cold stunning may occur from the misadventure of arriving in currents leading to colder waters. This is as opposed to sick animals that also strand during cold weather (Innis and Staggs 2017). In WA, sea turtles, predominantly post-hatchling loggerhead turtles, tend to strand in the South-West due to the prevailing currents and storms (Prince 2004). As these individuals are otherwise healthy, rehabilitation of these sea turtles can make a meaningful contribution to the recovery of populations which would otherwise not have survived (Spotila et al. 1997). This disorder has not been reported in leatherback turtles (which are relatively more cold tolerant) and larger sea turtles, which appear more able to cope with this disorder (Milton and Lutz 2003, Wyneken et al. 2006).

1.5.8 Infectious diseases

Few bacteria or fungi are primary pathogens, with most causing opportunistic infections and the majority of the reports are from sea turtles in captivity (e.g., sea turtle farms, or in rehabilitation) (Glazebrook and Campbell 1990a, George 1997, Innis et al. 2014). Gram-negative bacteria are most commonly associated with infection in sea turtles, with most

being normal flora for example, *E. coli, Salmonella* sp., and *Pseudomonas* sp. (Raidal et al. 1998, Ladds 2009). Systems usually affected include integumentary, respiratory, and skeletal, although systemic infections are also observed (Innis and Frasca 2017). While mycoses are uncommon in sea turtles, mycotic pneumonia is the most commonly reported fungal disease (Gordon 2005, Innis and Frasca 2017). The inability to cough (due to the lack of diaphragm and the presence of a hard shell), makes lung clearance difficult. Sea turtles are in general also prone to respiratory disorders (Boylan et al. 2017b). This is likely why pneumonia and other pulmonic disorders are so 'disastrous' in sea turtles (McArthur et al. 2004, Rodriguez et al. 2018).

Only four viruses have been detected in sea turtles to date, including herpesvirus, tornavirus, papillomavirus, and retrovirus (Page-Karjian and Herbst 2017). Clearly chelonian herpesvirus is the most common and important (see Section 1.5.2 – Fibropapillomatosis). Virology in reptiles is still in its infancy, with challenges associated with identification. However, molecular techniques are significantly improving our diagnostic capabilities along with our ability to discover new viruses (Marschang 2019), for example, a new papillomavirus has now been identified in Australia associated with FP (Mashkour et al. 2018).

For the vast majority of threats, the cause of infectious disease is through disturbance of the host-parasite-environment relationship, whereby changes to the environment (e.g., reduction of water quality or drop in temperature) may cause host immunosuppression, predisposing to opportunistic infection by pathogens to which they would not normally succumb. Even though these infections may not be primary pathogens, the disease caused by these agents can still be the primary diagnosis for the cause of morbidity and mortality. Gram-negative infections secondary to parasitism have caused mortality previously in sea turtles, and should be considered as a differential for morbidity and mortality (Gordon et al. 1998a, Raidal et al. 1998).

Whilst infectious diseases are increasing and emerging, so too is the reporting of novel pathogens and disease outbreaks caused by or associated with infectious agents in wild sea turtles (Gordon et al. 1993, Gordon 2005, Martinson et al. 2018, Young et al. 2020).

Microbiological and molecular diagnostics should be utilised to identify pathogens, including emerging diseases, and appropriate microbial treatment based on culture and sensitivity should be commenced as soon as possible.

Aside from the most significant parasites of sea turtles, the spirorchiids, sea turtles are host to a wide range of endo- and ecto-parasites. Many endoparasites including trematodes, nematodes, cestodes (intermediate stage), and protozoa, are specific to sea turtles and have co-evolved (Stacy et al. 2017c). Flukes (digenetic trematodes) are the most diverse group, requiring one or more other species to complete the life cycle. While the sea turtle is the definitive host, the intermediate host and parasite life cycle remain mostly unknown. While most nematodes are found in the alimentary system, sea turtles are the definitive host and as such they are generally not of clinical significance, except in cases of severe burdens (George 1997, Stacy et al. 2017c). Metacestodes/pleurocercoid larvae (trypanorhynch) are commonly found in sea turtles, an incidental finding, with most completing their life cycle in sharks (particularly tiger sharks) (Greiner 2013). While protozoa are relatively common in the GI tract of sea turtles, development of disease is uncommon. On the other hand, *Caryospora cheloniae* is a major pathogenic species responsible for coccidiosis (Leibovitz et al. 1978) which is suspected to be responsible for mass mortality events on the east coast of Australia, causing neurological and gastrointestinal signs, but to date disease caused by this protozoa has not been found in WA (Gordon et al. 1993). More recently, advances in molecular technology coupled with long-term health monitoring in the United States, have shown interoceanic dispersion of coccidiosis, now known as *Caryospora*-like coccidia, due to a lack of genetic material from the previous events (Chapman et al. 2016a, Stacy et al. 2019a).

While many endoparasite eggs and larval stages can be found on faecal floatation, eggs are often incidental findings and a sea turtle parasitology specialist is required to identify the species based on egg morphology (as for adult spirorchiids). However, molecular techniques have helped with these issues (Chapman et al. 2015). These parasites can be generalist or specialist in nature. Not unexpectedly considering their shared habitat, different sea turtle species can be affected by the same parasite species (generalists). However, green turtles,

particularly juveniles, are host to more parasitic species which are not found in other sea turtles (specialists). This is likely due to their ontogenic shift when they change from an oceanic carnivorous diet, to a predominantly herbivorous diet; this major shift can potentially disturb the host-parasite-environment balance and possibly expose them to more intermediate hosts (Stacy et al. 2017c).

The best way to determine the diversity and abundance of parasites is through necropsy of fresh sea turtle cadavers, specifically animals which died acutely and were otherwise healthy. While parasitism might be high in a population, sick animals have a tendency to skew the normal levels.

Sea turtles can host a wide range of epibionts, however barnacles are the most commonly reported (Stacy et al. 2017a). While some level of epibiosis is normal and there are some benefits to their presence such as camouflage, heavy burdens can increase weight and drag (Frick and Pfaller 2013). Excessive barnacles can also cause dermatitis with secondary infections while burrowing barnacles can penetrate bone leading to osteomyelitis (Boylan et al. 2017a). While the presence of epibiota does not necessarily indicate disease, Flint et al. (2010c) found sea turtles which had >20 individual barnacles on their plastron were more likely to be unhealthy. In flatback turtles, barnacles can become deeply embedded in the carapace, occasionally enabling the scutes to grow over them, which can subsequently rupture externally (potentially providing access for pathogen entry), and thus pose a possible cause of bony shell deformities (personal observation).

1.5.9 Zoonoses and public health

As previously mentioned, the health of sea turtles is of relevance to human health as demonstrated through the 'One Health' concept. Some bacteria such as *Salmonella* sp. are more common in captivity such as in sea turtle farms and pre-slaughter sea turtles, which could potentially pose a human health hazard in Australia (Ladds 2009). O'Grady and Krause (1999) reported a *Salmonella* sp. outbreak in an aboriginal community in the Northern Territory in 1988 causing GI signs following consumption of undercooked green turtle meat. Another concern with the consumption of a long-living animal, is the accumulation of toxins

and chemicals, with previous research finding high levels of cadmium in harvested sea turtles (Gordon et al. 1998b). Some protozoal parasites may also pose a potential zoonotic risk such as amoeba; however, most other endoparasites are for the most part host specific. Spirorchiid fluke eggs have been found in the faeces of aboriginal children, however as this parasite is host-specific, it is not a cause for concern (Blair and Miller 1992) as the infection is unlikely to transmit disease to humans (Blair and Miller 1992). Other common zoonotic reptile pathogens include *E. coli*, as well as *Vibrio, Pseudomonas, Aeromonas, Enterococcus, Mycobacteria, Fusarium, Candida, Entamoeba,* and *Cryptosporidium* spp. (Aguirre et al. 2006, Innis and Frasca 2017, Stamper et al. 2017).

1.6 General status and distribution of sea turtles in WA

Six of the world's seven sea turtle species occur in Australia, and all are found in WA waters, primarily in the Indian Ocean. These include the green, loggerhead, hawksbill, olive ridley, leatherback, and flatback turtle. Endemic to northern Australia, the flatback turtle is one of only two species without global distribution and was described as a separate species in 1988 (previously *Chelonia depressus* and *Natator tessellatus*). This was likely due to the remoteness of its home range (Bustard 1972). Identification of the various species is achieved by assessing the unique scale patterns and other anatomical features (Appendix 1 Indo-Pacific marine turtle guide). Important foraging and nesting grounds exist in WA which support some of the largest sea turtle populations in the world such as Ningaloo green, Gascoyne loggerhead, Rosemary Island hawksbill, and Pilbara islands flatback turtle (Limpus 2008a, b, 2009, Department of the Environment and Energy 2019f). All species, except the leatherback turtle, are known to nest in WA. Nesting occurs in the northern tropical parts of WA, with the most southern rookeries found around the Shark Bay and Denham area (Ackerman 1997, Cogger 2014, Department of the Environment and Energy 2019b) (Figure 1.6). Foraging hawksbill, olive ridley and flatback turtles are found in the warmer, subtropical and tropical northern parts of WA, while green and loggerhead turtles also utilise temperate habitats, seasonally dispersing throughout WA waters (Commonwealth of Australia 2017b, Witherington 2017) (Figure 1.5 and Figure 1.6).

1.7 Stranded sea turtles in WA

A stranded sea turtle is defined as any live or dead sea turtle found floating or washed up along the coastline, including tidal water inlets (Stacy et al. 2017b). As mentioned previously, aside from nesting females and the occasional basking turtle, sea turtles are not generally found on land except as hatchlings. In WA, recent legislation requires specially protected species (including sea turtles) to be reported within 24 hours (other wildlife is 72 hours) of being found or coming into care (*Biodiversity Conservation Regulations 2018*). There is a 'Marine Turtle Stranding Form' available to capture details of the stranding such as location, date, and animal status (Appendix 2 DBCA sea turtle stranding form), which is entered into the WA Stranding Database (WAStD), the register for stranded sea turtles (and other marine wildlife) in WA.

In WA, some species and life stages strand with seasonal patterns. Small neonate loggerhead turtles often strand following cold fronts blowing them onshore in the southern parts of WA. Other species and life stages strand randomly across the state with many predicted to go unreported because of the remoteness of the coastline (Dr S. Whiting, Department of Biodiversity, Conservation and Attractions, personal communication, May 2016). Although WA accounts for approximately one-third of Australia, it has one of the lowest human population densities in Australia, resulting in large stretches of uninhabited remote coastline with infrequent visitation. As such sea turtle and other marine wildlife strandings frequently go unreported or undergo advanced decomposition prior to discovery. This is in agreeance with Chaloupka et al. (2008) who found much higher reporting in populated areas. Challenges also revolve around logistical and financial constraints of recovering stranded dead sea turtles, especially large adults. Most information about sea turtle health and disease is derived from strandings, and equally, establishing cause of death is extremely important for understanding sea turtle diseases, with necropsy the single most useful way to investigate mortality (low-cost/low-technology) (Work et al. 2015). To address the issue of recovering dead sea turtles, freezers have been placed throughout regional WA at Department of Biodiversity, Conservation and Attractions (DBCA) compounds. While fresh cadavers are preferable, a frozen cadaver is favoured over a completely missed opportunity

to investigate cause of death. Sea turtle necropsy workshops have been held around the state, as an educational component of this project, to develop capacity in the regions to investigate sea turtle disease. Developing a functional stranding network is very important for WA, and while new legislation exists, it requires governing to ensure implementation.

There are currently five primary sites in WA with facilities for rehabilitating wild sea turtles (Dolphin Discovery Centre, Bunbury; Aquarium of Western Australia [AQWA], Perth; Native Animal Rescue, Broome; Ningaloo Sea Turtle Rehabilitation Centre, Exmouth; and Perth Zoo) and only one facility has on-site veterinary care (Perth Zoo). Fortunately, a second facility with on-site veterinarians is in the final stages of development (WA Wildlife, Bibra Lake, Perth), and should be operational early 2022. However, considering the large size of the state, there is a pressing need to further increase capacity for sea turtle rehabilitation within WA.

1.8 Aims and objectives

The status of sea turtle health in WA is largely unknown. Significant knowledge gaps exist with baseline health and disease levels, especially for flatback turtles. Furthermore, despite disease being frequently implicated as a contributing factor to sea turtle mortality, few detailed diagnostic investigations into morbidity and mortality have been undertaken in WA so the cause often remains unknown. Limited health-related research in WA has focused on sea turtle disease investigations (Edmonds et al. 1994, Raidal and Prince 1996, Raidal et al. 1998, Raidal et al. 2006), nesting loggerhead health assessments (Trocini 2013), and parasitological investigations (Lester et al. 1980, Blair and Limpus 1982), with stranding reports also contributing to our knowledge (Prince and Crane 1996, Prince 2004, 2007). There are also published blood RI papers from the east Indian Ocean for two green turtle populations and one hawksbill turtle population in Commonwealth waters (adjacent to WA waters) (Whiting et al. 2007a, Whiting et al. 2014a). In other parts of Australia, many baseline blood parameter studies exist (Hamann et al. 2006, Whiting et al. 2007a, Flint et al. 2010b, Flint et al. 2010c, Whiting et al. 2014b), as well as numerous investigations into

disease and causes of sea turtle mortality (Glazebrook and Campbell 1990b, Flint et al. 2010c, Flint et al. 2015b).

The flatback turtle is the least studied species of sea turtle, with only one physiological study published (Sperling et al. 2007) and no publications existing on disease and mortality studies including parasitology (Prof. D. Blair, James Cook University, personal communication, 17 April 2017), aside from reference to occasional observations such as flatback turtles observed with fibropapillomas on Crab Island, Queensland (Limpus et al. 1993). The only previous blood work for flatback turtles focused on blood indicators of stress associated with various handling techniques (Guinea 2016). More recently, the relationship between microbiome and blood values (or lack thereof) has been examined in flatback turtles (Scheelings et al. 2020), however in comparison to other sea turtle species, the flatback turtle currently remains poorly understood. It is therefore imperative to prioritise further study on flatback turtles (Deem and Harris 2017).

This WA sea turtle research project aims to establish health and disease baseline data, as well as establish the major causes of stranding of sea turtle species through morbidity and mortality investigations, with the main overall objective being to develop a greater understanding of sea turtle health in WA. While sea turtles from all over WA were utilised in the stranding investigations, specific rookeries and foraging grounds (predominantly those utilised by flatback turtles) were targeted for development of baseline blood RIs. The project was undertaken over a three-year period, and represents the first state-wide, systematic health and disease investigation of sea turtles in WA.

There were two major objectives to this research project, each with several main hypotheses.

1. Establish a health and disease baseline for sea turtle species, primarily flatback turtles, in WA.
    a. Baseline health and disease levels evidenced through haematological and biochemical analysis, are markedly different between nesting females at the nesting beach, as compared to those captured on foraging grounds.

b. Baseline levels of health and disease represented by blood RVs are the same between different nesting rookeries.
c. Results from field-based analytical techniques are comparable to those obtained from commercial-based labs.

2. Assess the causes of morbidity and mortality of sea turtles in WA.
   a. Disease-related causes of morbidity and mortality for sea turtles in WA are significantly higher than direct anthropogenic causes.
   b. Spirorchiid infection is a significant cause of mortality for all sea turtle species in WA.
   c. Spirorchiid parasites infecting sea turtles in WA are the same species and genera to those on the east coast of Australia.

1.9 Thesis structure and outline

This thesis is divided into six chapters, one of which is based on a modified version of the published paper (Young et al. 2020). Chapter one is an introduction to sea turtles including their biology, physiology, conservation status, and threats, with a particular emphasis on health, disease, and sea turtle strandings in WA. Chapter two covers the general methodologies used to establish baseline health RIs for flatback turtles and disease investigations, including necropsies, for stranded sea turtles in WA and ancillary diagnostics utilised to reach a diagnosis. Chapters three and four focus on flatback turtle RIs, and morbidity and mortality (including parasites) of sea turtles in WA respectively. Chapter five is a modified version of the paper on the marine wildlife mass mortality event in north-west WA in March 2016. The final chapter, Chapter six, is a general discussion and concludes the thesis by summarising the major findings and their implications and finally proposes recommendations (including veterinary management and rehabilitation) for the conservation and management of sea turtles in WA.

CHAPTER 2

General methodology

This chapter describes the study area and animals, general methodology of the veterinary examination (or necropsy examination in the case of dead sea turtles), and sample collection and laboratory tests used to investigate the health and disease status of sea turtles in WA. Standardised methods were utilised, where appropriate, to facilitate comparisons with previous studies undertaken elsewhere in Australia or overseas.

Additional methodology specific to the research discussed in each chapter such as statistical analyses, can be found in the methods section of the chapters.

2.1 Developing a health baseline

2.1.1 Study sites and animals

The health baseline research was conducted at three study sites including two flatback turtle rookeries (Eighty Mile Beach and Thevenard Island) and two flatback turtle foraging grounds (Roebuck Bay and Eighty Mile Beach) (Figure 2.1). Gravid mature females were sampled at the rookeries they inhabited, while sea turtles from various sex and age-class groups were sampled at the foraging grounds. The selected study sites allowed for comparison of blood results between rookeries, as well as between nesting and foraging turtles while providing different study area categories based on their locality or close proximity to sites including undeveloped (Eighty Mile Beach), industrialised (Thevenard Island) or urbanised/town (Roebuck Bay). However, it is important to note the relatively transient periods spent in the general vicinity of the rookery as reflected by the cyclical nature of a sea turtle's life history (see Chapter 1.6 – General status and distribution). By satellite tracking a subset of nesting turtles (n=12), potential foraging grounds could be identified, which theoretically represented the animal source more accurately by identifying the turtles' more permanent origins. These rookeries were selected based on advice by DBCA, due to the sites being medium density nesting sites. As for the foraging areas, these are the only two known sites where flatback turtles are accessible in the foraging grounds (Dr S. Whiting, DBCA, personal communication, 4 May 2016). Each study site is described in detail below.

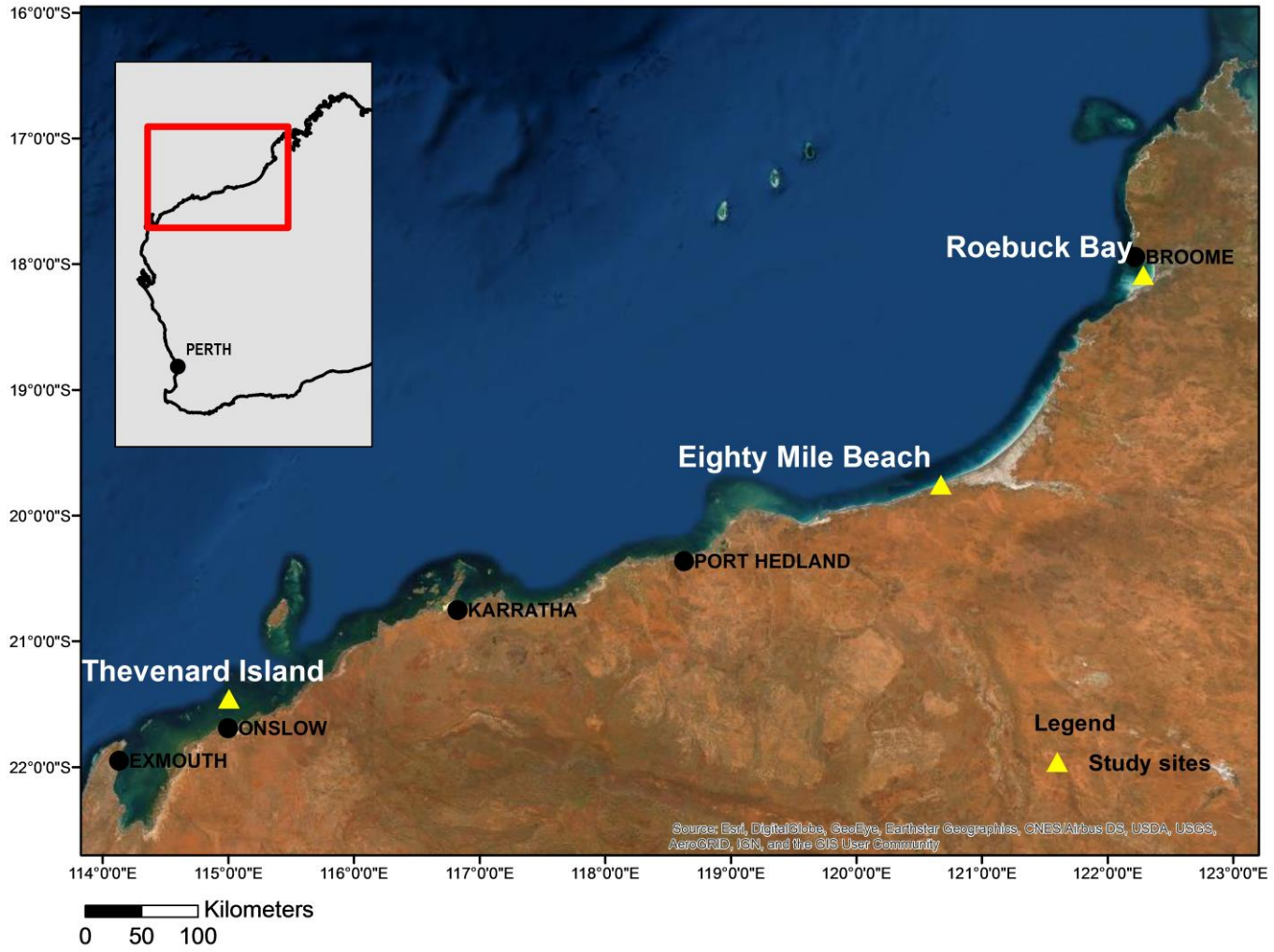

Figure 2.1 Map of nesting and foraging sites for flatback turtles sampled in Western Australia.

Eighty Mile Beach

Located between Broome and Port Hedland, Eighty Mile Beach is a 220 km long stretch of undeveloped and remote coastline which lies within the Eighty Mile Beach Marine Park, jointly managed by DBCA together with the Nyangumarta, Karrajarri and Ngarla Traditional Owners (Figure 2.2). Not only is Eighty Mile Beach an important flatback rookery, adjacent waters are also significant foraging grounds for many sea turtle species, including flatback turtles, identified through satellite tracking (Seaturtle.org 2018a), it is also a Ramsar listed wetland of international significance for migratory shorebirds of the East Asian-Australasian Flyway (EAAF) (East Asian-Australasian Flyway Partnership 2013).

Eighty Mile Beach consists of an almost continuous sandy beach, low dune system, extensive intertidal zone (up to 4 km wide), and lies within a large tidal range region (~10 m) (Department of Parks and Wildlife 2014). The topography is a gentle sloping beach, with intermittent sections of exposed beach rock (limestone), and depending on current sand movement, the high tide contacts the dunes leaving a very narrow strip of usable nesting habitat for the flatback turtles. Various research and monitoring programs undertaken at this beach include track counts, nest exhumations (for nesting and hatching success), satellite tracking, stable isotope analyses, and aerial surveys. It is a popular fishing recreation area during the dry season but fortunately, low recreational visitation during the wet season coincides with flatback nesting season. However, the beach is situated in an area of high cyclonic activity, and cyclones, storm surges, and low-pressure systems can result in significant damage to the nesting beach. Nevertheless, compared to many other nesting beaches, minimal impacts, particularly anthropogenic, exist for this beach and the nesting turtles.

Eighty Mile Beach is considered a medium density flatback nesting rookery, and while nesting is distributed along the length of this beach, higher nesting activity occurs around Wallal Downs and Anna Plains (Figure 2.2 and Figure 2.3).

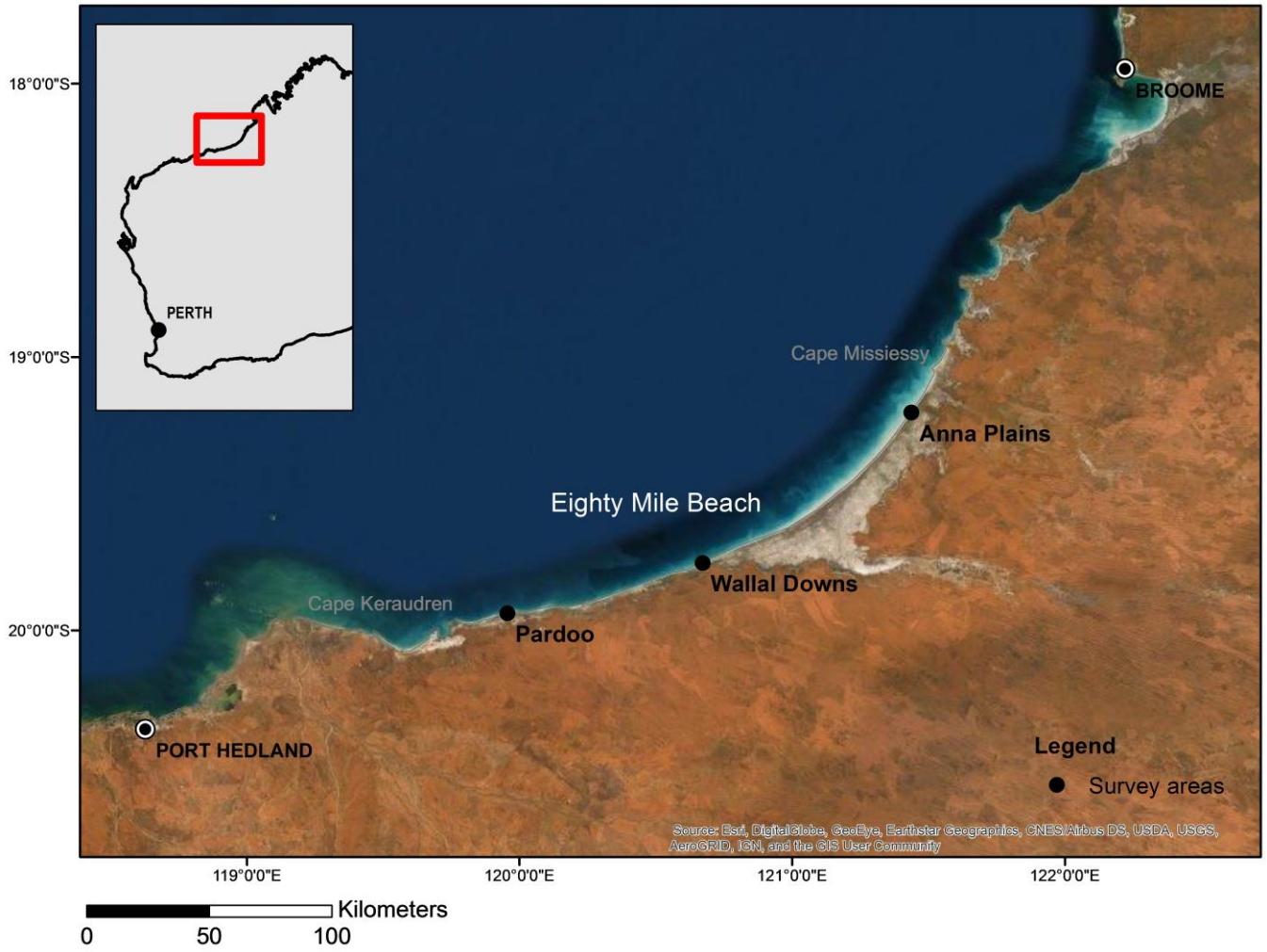

Figure 2.2 Eighty Mile Beach study site (from Cape Missiessy to Cape Keraudren) with survey areas.

Figure 2.3 Eighty Mile Beach Wallal Downs survey area during nesting season.

Thevenard Island

Thevenard Island is a small remote island (5.5 km^2) in the Pilbara 25 km north-east of Onslow and is a low, relatively flat, vegetated island, surrounded by limestone reef, with surrounding shallow bathymetry (Chevron 2017). It has mixed tenure including a tourist resort lease (Mackerel Islands resort), a mining lease in the process of being decommissioned (Chevron) and DBCA managed estate (Figure 2.4 and Figure 2.5). This C class nature reserve was identified as an important flatback rookery and selected for a 30-year monitoring program as an offset of the North West Shelf Venture, the largest resource development project in Australia (North West Shelf Gas, 2018). Despite the close proximity to major oil and gas projects (Wheatstone 25 km to the south-west of Onslow and Gorgon on Barrow Island, 90 km to the north-east), and previous on-site mining facilities, Thevenard Island serves as a useful site for comparison with these industrialised sites, particularly Barrow Island, as both support important nesting sites for flatback turtles, as well as low density green and hawksbill turtle nesting sites.

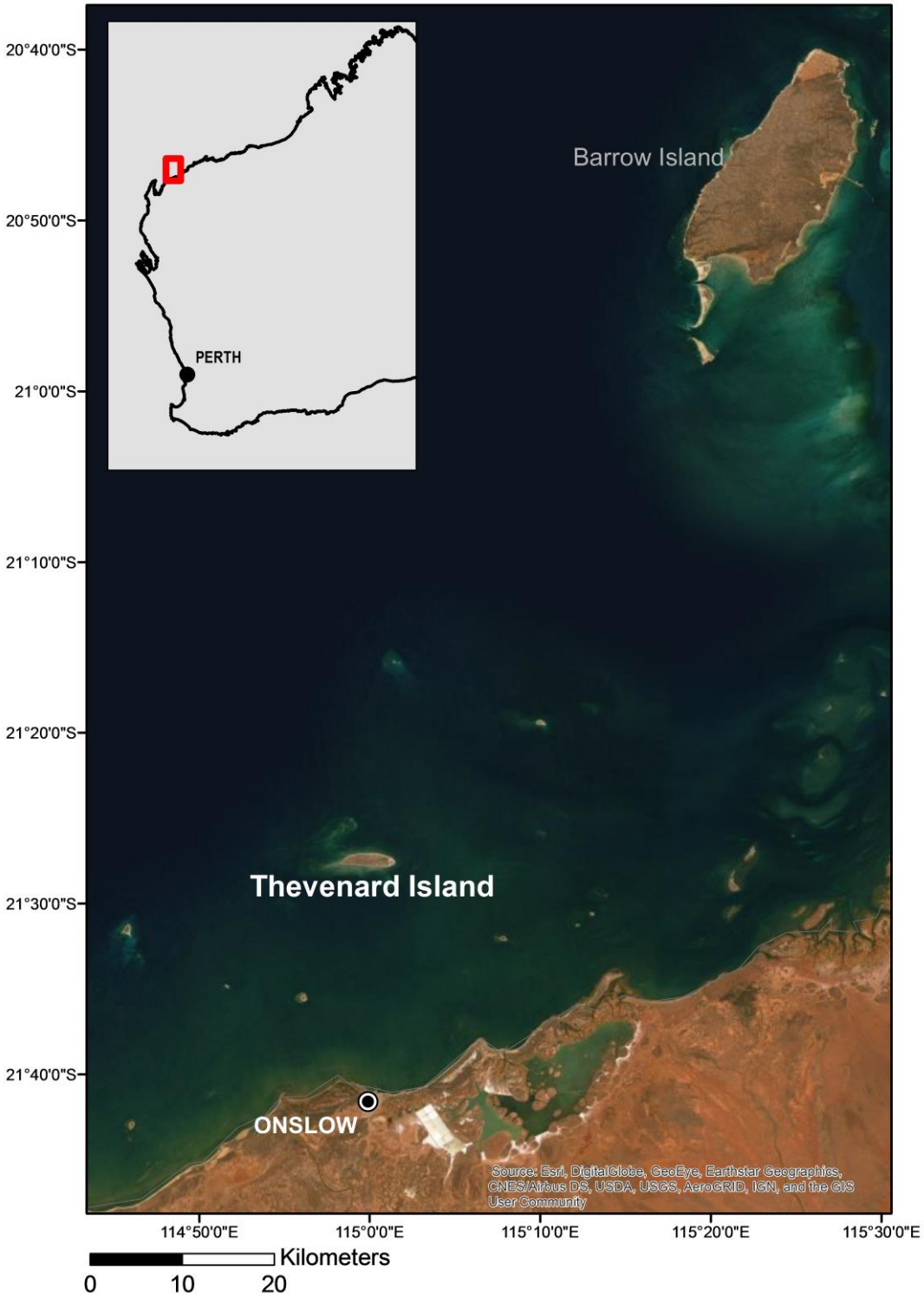

Figure 2.4 Thevenard Island location.

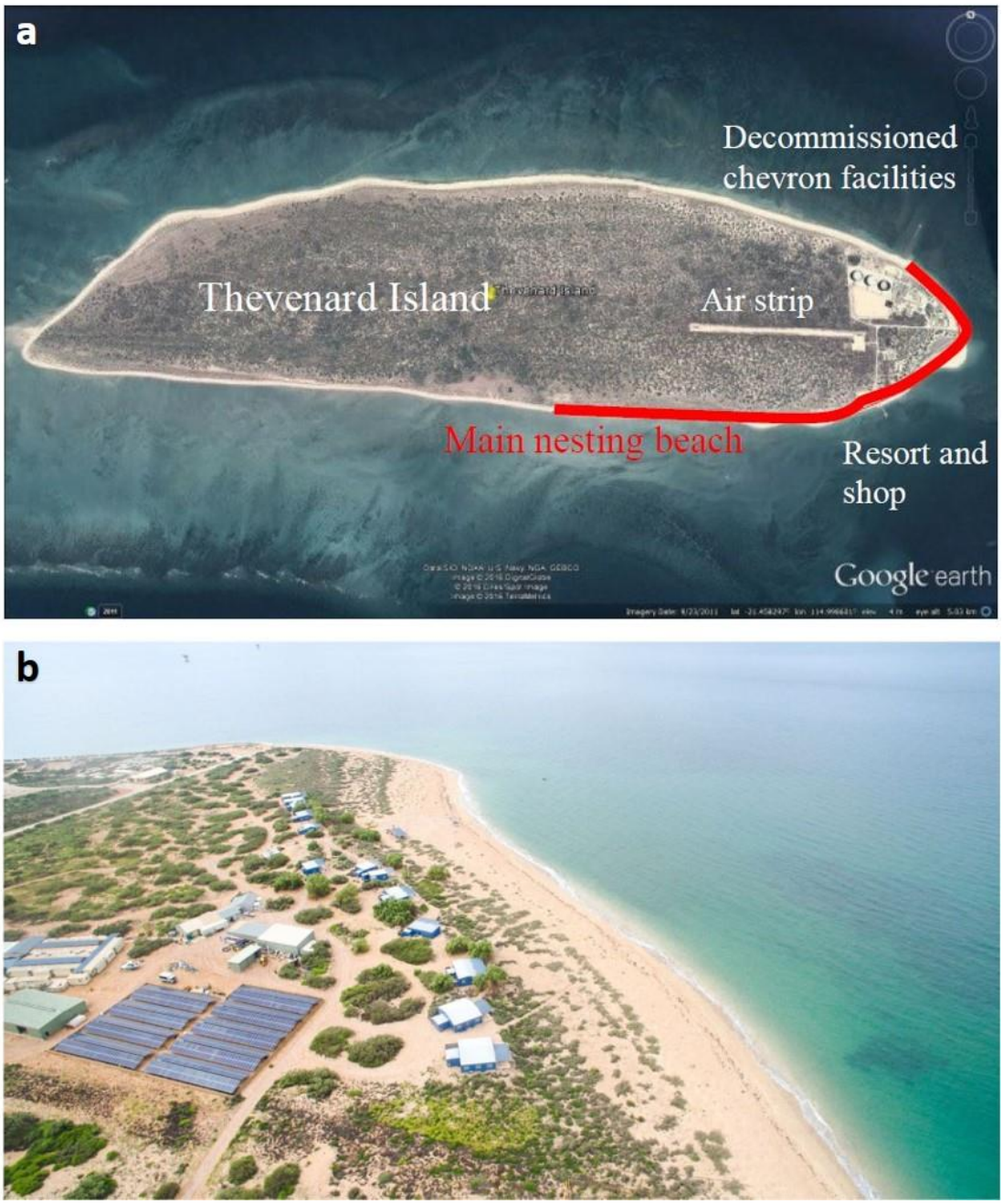

Figure 2.5 Aerial views of (a) Thevenard Island, and (b) Main flatback nesting beach north-eastern side of Thevenard Island (Department of Biodiversity, Conservation and Attractions 2018).

In 2016, DBCA commenced monitoring this rookery, and various research and monitoring programs have since been instigated including track counts, nesting success, nest exhumations, logging sand temperature, aerial surveys, satellite tracking, and recording sea turtle nesting activities in daily diaries. Through this monitoring,

several nesting flatback turtles previously tagged on Barrow Island were found attempting to nest on Thevenard Island in subsequent seasons which could be a normal phenomenon that has been detected through monitoring of a new site, or could possibly related to changes in their former nesting beach due to the construction of a jetty structure. Artificial light both on land and in-water poses the most serious threat for hatchlings (Wilson et al. 2018).

Roebuck Bay

Roebuck Bay is the bay adjacent to Broome townsite and in 2016 became the jointly managed Yawuru Nagulagun/Roebuck Bay Marine Park by DBCA and Yawuru Traditional Owners (Figure 2.6). This site was selected as it had been identified as a hotspot for foraging flatback turtles through local observations and satellite tracking data from nesting flatback turtles.

Roebuck Bay is a listed Ramsar wetland site and is a highly important area for migratory shorebirds. It is considered a multi-use marine area and is recognised for its cultural, ecological, commercial, and recreational values which include aquaculture (Paspaley Pearl farm lease), a commercial port, recreational fishing (commercial fishing ceased in 2013), (eco)tourism, and the Yawuru people's strong connection to their land and sea country (Department of Parks and Wildlife 2016).

Roebuck Bay is 550 km^2, with shallow bathymetry, a large tidal range (up to 10 m) with an intertidal zone up to 12 km in some parts. The mudflats, creeks, mangrove stands, and seagrass meadows provide important nurseries, habitat, and food which supports species of high conservation value making it one the most productive places on earth (Roebuck Bay Working Group 2011). Current research undertaken in Yawuru Nagulagun/Roebuck Bay Marine Park includes benthic surveys on mudflats, seagrass monitoring, shorebird counts, sea turtle monitoring, and habitat mapping of the Bay.

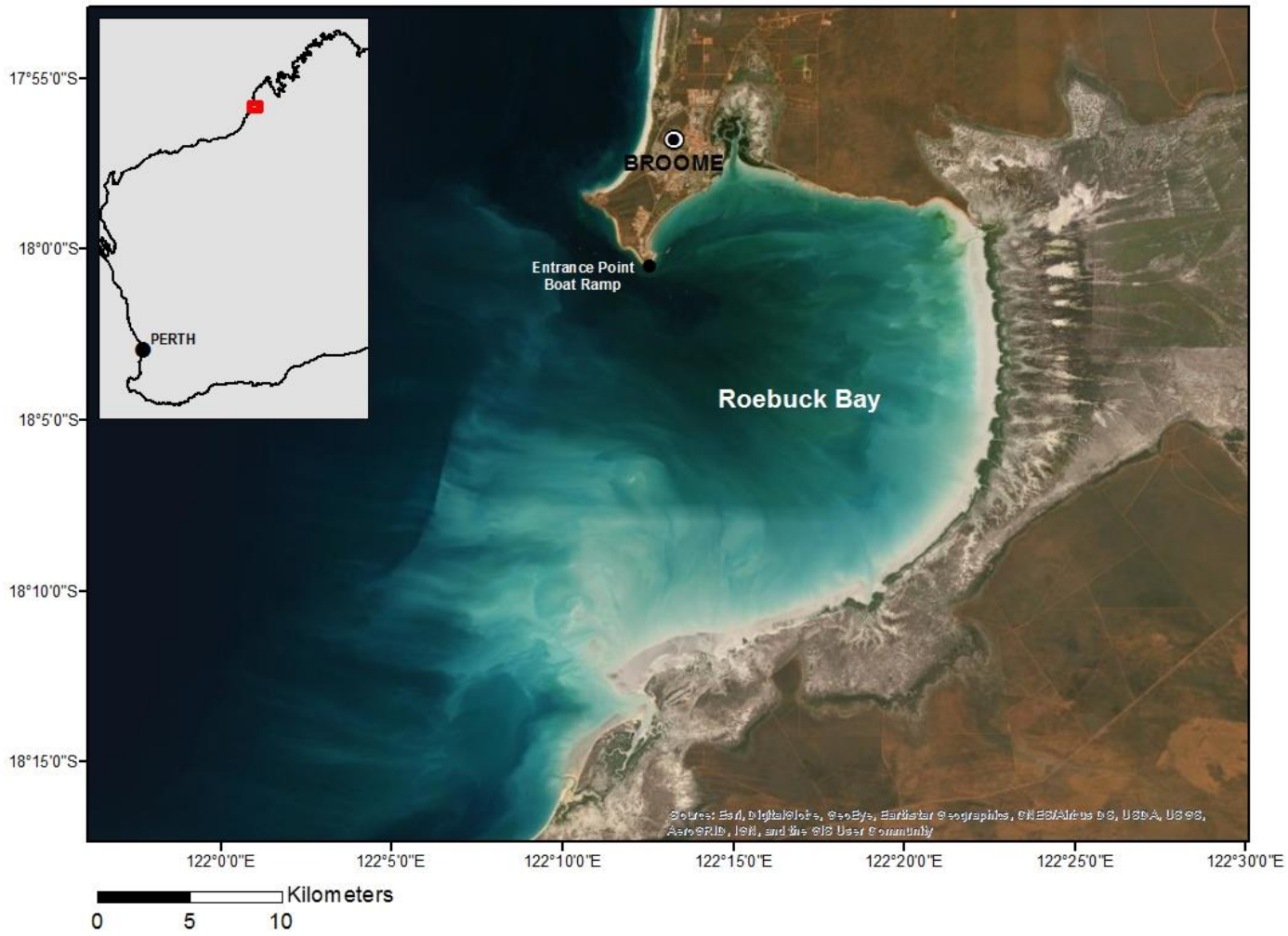

Figure 2.6 Map of Roebuck Bay.

2.1.2 Survey techniques

Study animals and seasonality

Nesting females were assumed to represent a healthy group of animals (single age-class and sex), albeit with potentially altered blood values related to reproductive status. Conversely, sampling at the foraging ground during the non-breeding season, provided samples from both sexes, different size/age classes, and non-breeding females, and therefore blood values were considered representative of a non-breeding flatback turtle. All sampling was opportunistic and further details of the survey methods undertaken at the rookeries and foraging grounds are described below.

Rookeries

Biological samples were collected from 148 flatback turtles during six field trips over two seasons and 31 sample nights. The majority of these trips were undertaken during peak nesting season (November-December) at Eighty Mile Beach (14-16 November and 6-12 December 2016, 10-13 December 2017) and Thevenard Island (20-25 November 2016 and 20-28 November 2017), while one trip to Thevenard Island occurred at the end of 2016/17 nesting season (2-6 February 2016). Seventy-four flatback turtles were sampled over the three field trips at both Eighty Mile Beach (n=8, 38, 28 respectively), and Thevenard Island (n=26, 12, 36 respectively). However, three flatback turtles were sampled twice, on two occasions inadvertently on Thevenard Island within four days (these samples were excluded from RI analyses) and once at Eighty Mile Beach almost one year apart (included in RI analyses). Therefore, the number of flatback turtles from which samples were analysed from Thevenard Island was 72 (n=25, 12, 35 on the respective trips) while the number of flatback turtles from which samples were analysed at Eighty Mile Beach remained the same (n=74).

Three different survey areas were utilised at the Eighty Mile Beach study site with sampling predominantly undertaken within the 6 km higher nesting activity Wallal Downs survey area (n=71/74) (at this location there were an average of 50 nesting

activities/km/recorded per night). Two turtles were also sampled on the first Eighty Mile Beach field trip at Anna Plains in a 20 km survey area as well as one turtle from Pardoo, a 10 km survey area at the southern end (Figure 2.2). While on Thevenard Island, all flatback turtles were sampled at a single 1.5 km long tagging survey area on the north-eastern side of the island (Figure 2.5b).

At Eighty Mile Beach, rangers, Traditional Owners, station-hands from the local stations, caravan park staff, visitors and residents, and other volunteers assisted with the surveys. While at Thevenard Island, DBCA staff, other researchers, and trained volunteers assisted.

Eighty Mile Beach was surveyed by 4WD-utility vehicle whilst Thevenard Island was surveyed by foot. At Eighty Mile Beach, due to the expansive mudflats and large tidal range, nesting patterns are strongly influenced by tides and monitoring was undertaken two to three hours before and after high tide during twilight hours (generally between 1800-0600 hours), and generally involved six hours monitoring effort per evening. At Thevenard Island, while peak abundance was around high tide, flatback turtle activity was more dispersed than at Eighty Mile Beach. This was possibly related to a smaller tidal range and bathymetry adjacent to the nesting beach, which meant nesting could occur at virtually any tide. At Eighty Mile Beach, we would drive along the beach (in one convoy if multiple vehicles) above the high-water mark until we encountered an emergence track or a sea turtle returning to the ocean (preferably post-lay). On Thevenard Island, DBCA staff and volunteers would walk the beach in small groups until an emergence track or sea turtle was encountered. At both rookeries, we would follow the track/sea turtle, following the Turtle Watcher's Code of Conduct (Department of Parks and Wildlife 2013b) so as not to disturb the turtle, and determine the stage of nesting. Depending on the number of assistants we would then either wait until the sea turtle had finished nesting and was returning to the ocean or, leave an assistant with a radio to communicate when the sea turtle was returning to the ocean. Details of the capture and restraint techniques associated with sample collection are described in Section 2.1.3.

Foraging grounds

We sampled 43 foraging flatback turtles during two field trips in the first season to Roebuck Bay over 10 capture days (25-29 June and 27-31 August 2018). On the first trip we captured 22 flatback turtles and on the second trip we captured 21 flatback turtles (one recapture), one olive ridley turtle and one green turtle. As the individual sampled twice was sampled within three months and appeared in less than optimal health on the second trip (average body condition, higher than normal barnacle load, and multiple open wounds), the second sample was excluded from the analysis, due to sample frequency and clinical findings. An additional seven flatback turtles, as well as 19 green and three olive ridley turtles were sampled on a third foraging trip in the second season over five capture days (flatbacks captured 4/5 days) to Eighty Mile Beach Marine Park (27 April – 5 May 2019). The total number of foraging flatback turtles from which samples were used for analyses was 49 (n=22, 20, 7 on the respective trips).

Two DBCA vessels were used to access the Roebuck Bay foraging grounds (Figure 2.7). Jangabarri is a 5.8 m Naiad and a purpose-built patrol vessel. Linygurra is a 5.5 m purpose-built work vessel to carry out research (e.g., benthic surveys, crocodile population surveys and removal). Each day eight to 10 personnel including Parks and Wildlife marine rangers, wildlife officers, research scientists along with Yawuru rangers, Traditional Owners and other researchers partook in these surveys. Most days we departed mid-morning (0930-1000 hours) and returned in the afternoon (1600-1700 hours), spending approximately six to seven hours on the water, with approximately 15-20 minutes travel to and from the survey area. While we managed to access the bay every day, certain weather, including strong winds and swell complicated sampling. We observed that days with a slight wind resulted in higher numbers of sea turtles sampled, as on very calm days the flatback turtles were more aware of vessel approach and harder to catch. Irrespective of tide and other weather conditions, all but one flatback turtle was captured before noon. At the time of

capture, flatback turtles appeared to be basking, positively buoyant and presumably following blooms of jellyfish.

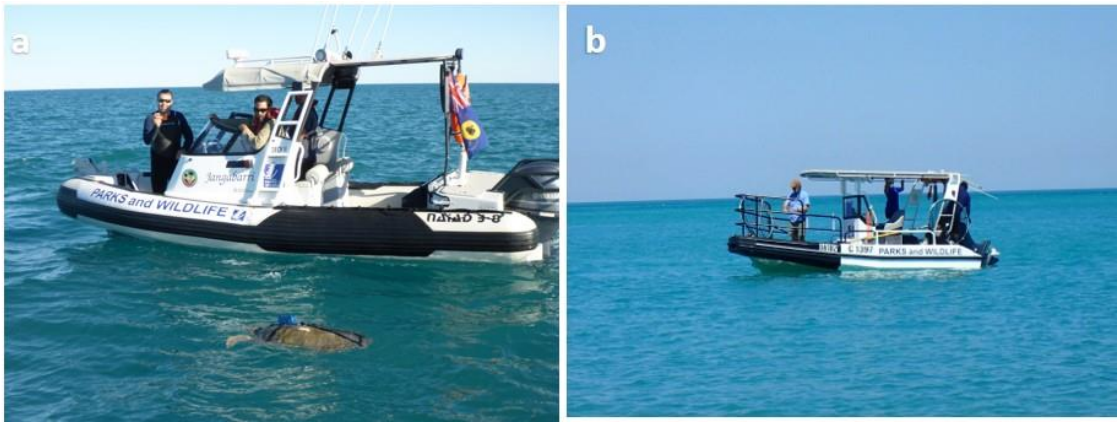

Figure 2.7 Roebuck Bay in-water flatback turtle research vessels showing (a) Jangabarri, and (b) Linygurra.

We travelled to an area of Roebuck Bay known to the locals as 'Middle Ground' approximately 2 km due east from the vessel launching area at Entrance Point (Figure 2.6), which has previously been identified as a flatback turtle 'hotspot'. According to the senior Marine Park ranger and head skipper, the surveyed area (~60 km^2) had an average water depth of 30 m and a sea floor comprising a mix of reef structure and sandy bottom (Mr A. Richardson, DBCA, personal communication, 28 August 2018). The survey involved the two vessels entering the area within 500 m of each other to spot sea turtles. Once a sea turtle was caught the skipper would communicate with the other vessel by radio and the two vessels would raft up together in order to process the animal. Frequently, two sea turtles were caught almost simultaneously, and in these instances, the turtles were processed concurrently on the vessels. Further capture details are provided in Section 2.1.3.

On the Eighty Mile Beach foraging trip, we stayed on the DBCA liveaboard, Worndoom, the patrol vessel for the Lalang-garram Marine Park which is jointly managed with the Dambimangari Traditional Owners (Figure 2.8). As this was the first foraging turtle study within the Eighty Mile Beach Marine Park, we surveyed various sites, encountering no turtles on some survey days. From Broome, we motored to the

southern part of the Marine Park (~350 km) where we searched for turtles using the two on-board tenders (small vessels) surveying in a similar fashion as described above, encountering juvenile green turtles at Cape Keraudren (Figure 2.2). We travelled north the following day and found foraging flatback and olive ridley turtles in the waters adjacent to Wallal Downs and Anna Plains, before returning to Broome. Nyangumarta and Ngarla rangers and Traditional Owners assisted with this research, along with DBCA Marine Science Program, Eighty Mile Beach, Yawuru Nagulagun, and Lalang-garram Marine Park staff.

Figure 2.8 Worndoom Department of Biodiversity, Conservation and Attractions (DBCA) liveaboard patrol vessel, Eighty Mile Beach Marine Park.

At all sampling sites, additional and relevant environmental and ocean conditions were recorded (when available) for potential inclusion in the statistical analyses for the blood RIs including air and sea surface temperatures, sea state and other local weather conditions.

2.1.3 Capture and restraint

Prior to being captured, sea turtles were checked for flipper and/or microchips/pit tags (LID 573 portable multireader, Trovan®, UK) to avoid duplicate sampling. Flatback turtles at Eighty Mile Beach were only pit tagged, except during the first field trip

undertaken in conjunction with DBCA staff who flipper tagged 6/8 sampled flatback turtles. Nesting flatback turtles selected for sampling were returning to the ocean, preferably post-lay, but turtles which false-crawled (defined as an unsuccessful nesting attempt) or whose nesting success was unknown were also selected for sampling. All attempts were made to ensure unconfirmed nesters were given the opportunity to re-attempt nesting at this location and only captured once their behaviour indicated they were returning to the ocean.

Nesting flatback turtles

Restraint techniques commonly utilised by DBCA to deploy satellite trackers, namely by placing the sea turtle onto a 'nally tub' (heavy duty polypropylene plastic box) were initially deemed unsuitable. It was difficult to immobilise a strong and heavy animal (<100 kg) for blood collection on the tub and this restraint technique also obscured examination of the plastron (lower shell). Therefore, an improved technique, involving the modification of a wheelbarrow to precise specifications was built to restrain flatback turtles for blood collection, which is a restraining device frequently used for sea turtle laparoscopy (Dr S. Whiting, DBCA, personal communication 18 November 2016) (Limpus and Reed 1985a). As assistants became more experienced in sea turtle restraint, and blood collection efficiency improved, placement on a nally tub subsequently proved useful for restraint (see Foraging flatback turtles below).

Turtle restraining device or 'turtle barrow'

The turtle restraining device (TRD), otherwise known as the 'turtle barrow' was a modified wheelbarrow with a steel frame and wooden handles (Sherlock 100L Trade Tough Steel Tray https://www.bunnings.com.au/sherlock-100l-trade-tough-steel-tray-wheelbarrow_p3350147). Construction involved the removal of the steel tub, and the front supports followed by the subsequent shortening, angling and reattachment of these supports. This was in combination with the application of piece of timber (which served as a stop to prevent forward movement of the sea turtle), as wide as the handles, to the upper surface of front end of the handles. The final step involved

the attachment of a polygonal piece of acrylic 38cm in length, 30cm wide at base, tapering to 26cm at front end of wheelbarrow which provided a flat base at the front of the wheelbarrow to support the head/neck of the sea turtle and stop the tyre rubbing against the head when moving the turtle barrow. Soft ropes were then securely attached to the frame of the wheelbarrow beneath the back edge of the acrylic (used to diagonally cross over, tie to the opposite handle and hold the sea turtle in place). All sharp edges were filed, and hard surfaces protected with soft padding. A second replica TRD was constructed in order to station a TRD at each rookery (Figure 2.9).

Figure 2.9 (a) Turtle restraining device (TRD), also known as the 'turtle barrow', and (b) TRD in use in the field.

At both sites female flatback turtles were captured when returning to the ocean, preferably post-lay. Sea turtles were captured by hand on the beach, usually by two to four people. The sea turtles were either lifted by grasping the carapace and placing directly onto the TRD. Alternatively, with the TRD placed in front of the sea turtle (front of the wheelbarrow pointing towards the ocean) the TRD was tilted so the handles angled downwards and contacted the ground. Two handlers approached the sea turtle and placed nooses made from soft rope around the proximal aspect of each of the front flippers. With one person on either side grasping the rope/carapace and one at the back of the carapace, the flatback turtle was then slid/lifted onto the TRD in a smooth gliding motion (Figure 2.10).

Once the flatback turtle was in the correct position with its plastron up against the padded wooden bar and head/neck overlying the acrylic surface, the TRD was tilted back to the horizontal position and the two ropes at the front of the TRD were quickly passed over the sea turtle diagonally to the TRD handle of the opposite side, to cross mid-carapace and were then tied firmly to the handles at the back of the shell (Figure 2.10b). With the flatback turtle firmly secured in position blood collection commenced (see Section 2.1.5 below for further details of blood collection).

Figure 2.10 The TRD in action showing (a) Capture technique, and (b) Restraint.

Occasionally, nesting flatback turtles were sampled without restraint/very gentle head restraint during the covering up stage of the nesting process. While this variation of animal handling had the benefits of reduced stress for the animal and potentially less impact on blood analytes, the timing for such sampling was crucial otherwise it could lead to abandonment of the nesting process and a very difficult and agitated animal to handle and sample (see Section 2.1.5 for more details on sample timing). Although it seemed that restraint on the TRD may cause some temporary stress, it appears this technique was preferred for both the safety and security of sea turtles and humans.

The objective of the Thevenard Island tagging program is to tag all nesting females for mark-recapture estimates. Due to the propensity for flatback turtles to simultaneously emerge from and return to the ocean (usually associated with high tide but potentially related to other environmental cues or conspecific communication such as in arribada – see Section 1.3), multiple groups of volunteers surveying the site every 20-30

minutes increased beach coverage and facilitated an increased chance of sea turtles being caught simultaneously. This simultaneous emergence led to the need for one sea turtle to be held temporarily, while blood was collected from the turtle which had been caught first. On these rare occasions, the second turtle was placed on a nally tub until the procedure was completed on the first turtle which did result in some turtles being held slightly longer than 20 minutes, however, after initial capture, most turtles would typically stop struggling within 10 minutes. Only one sea turtle would be held while another was sampled, and this seemed a good compromise between missed sampling and the welfare considerations of the animal. Given the sampling was opportunistic on a remote island and with a limited sampling period, this strategy ensured samples collected were sufficient to warrant an economically viable and scientifically sound field trip.

Foraging flatback turtles

Two techniques were utilised to catch foraging sea turtles. The first was using the rodeo method from the research vessels or the tenders (Limpus and Walter 1980) (Figure 2.11). Once a sea turtle was spotted, the turtle was approached at a steady speed while the catcher was positioned at the bow. Every attempt was made to approach the sea turtle from behind and at a distance approximately 5-10 m from the turtle, the vessel was placed in neutral, using momentum to reach the turtle (with occasional repositioning required). With the sea turtle positioned to the side of the bow (10 or 2 o'clock position) approximately 1-2 m away, the catcher would jump from the bow onto the turtle floating at or just below the surface, grasp the carapace, and quickly flip vertically (head up) ensuring its powerful foreflippers were out of water. Using nooses, the crew on the vessel would grasp the flipper and pass the noose to the base of the foreflipper and then tighten. Simultaneously, the catcher and crew on the foreflipper ropes would heave the animal onto the side of the vessel, and then lower it to the floor of the vessel (covered with rubber matting) (Figure 2.12). Alternatively, a custom-fabricated dip net with a 2 m fiberglass handle, 1 m aluminium diameter hoop, and a 2.5 m long bag of soft mesh was also used to scoop sea turtles

from the bow of the patrol vessel (Figure 2.11b) (Dr A. Tucker, DBCA, personal communication, June 2018). This involved a similar vessel approach. The net was held by the catcher on the bow and would be passed over the front of the sea turtle, as the turtle swam further into the net. This was the theory, however sometimes the sea turtle spun around inside the net which complicated the capture. Once a sea turtle had been successfully caught in the net, the turtle would be hauled around to the side entry and onto the vessel. The sea turtle would be removed from the net and was placed on the floor of the vessel for sampling. Overall, this latter capture technique had a lower success rate but became the primary capture method after a risk analysis deemed it safer for personnel due to the known presence of dangerous animals within survey areas including tiger sharks, crocodiles, and jellyfish. Further, lifting a turtle into the vessel by its fore flippers with ropes following rodeo capture is standard practice and generally does not cause problems in healthy, robust turtles. However, this technique can cause fractures and other musculoskeletal trauma, as such using the net also improved the animal welfare outcome. Foraging flatback turtles were generally held for approximately 30 minutes, except when additional research and procedures were performed such as satellite tracker deployment.

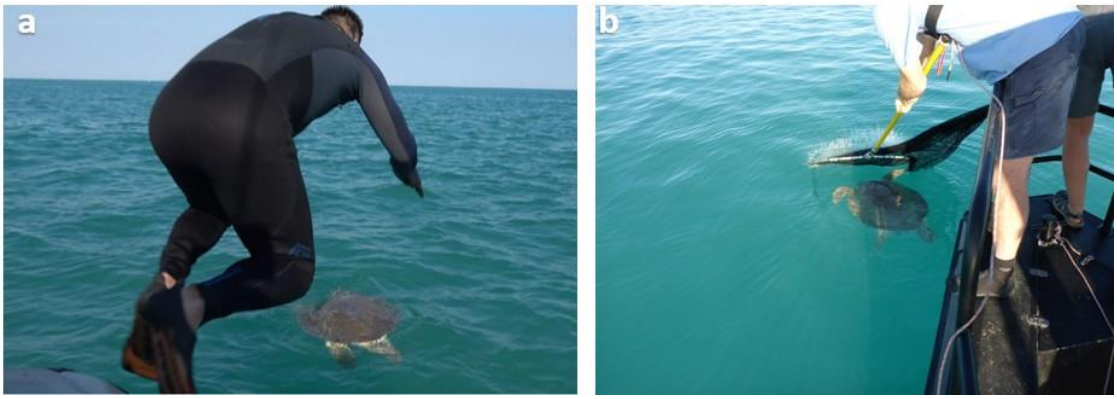

Figure 2.11 Foraging flatback turtle capture by (a) Rodeo, and (b) Dip net (photos courtesy of DBCA).

The method of restraint for foraging sea turtles varied depending on the particular animal's mentation. In the first instance, minimal restraint was attempted, followed by the addition of a head holder, then additional restraint of body, and lastly for more

active animals, they were placed on a nally tub plastron down, with flippers positioned around the tub to minimise any purchase on the tub. Additional crew would stabilise the sea turtle on the tub and an additional head holder would be utilised to significantly minimise movement (see Section 2.1.4 below).

Often the working space on the tenders was limited, so working on the animal without placing them on the tub was less complicated (Figure 2.12) but sometimes necessary as explained above. In contrast, on the liveaboard Worndoom, there was ample working space.

Figure 2.12 (a) Flatback turtle placed on the vessel floor for sample collection, and (b) Confined working space on vessels.

2.1.4 Health assessment

A health assessment was performed on each individual caught for sampling and involved a basic external physical examination; an assessment of body condition; recording, classifying, grading, and estimating the duration of external abnormalities; documentation of presence, burden, and coverage of epibiota including barnacles and algae; neurological examination including evaluation of mentation; and musculoskeletal examination including assessing mobility (Figure 2.13) (Flint et al. 2010c). In order to ensure consistency, all assessments were performed by the author. Examination and morphometrics including weight, curved carapace length (CCL), curved carapace width (CCW), along with carapace to tail (*CT*), plastron to tail (PT), and vent to tail (VT) in males, were performed concurrently with procedures for other

projects, such as biopsy sampling and satellite tracking, but these were all generally conducted post-blood collection. The TRD facilitated examination of the animal's ventral surface i.e., tail, plastron, and cloaca, and this was the primary technique used to examine the majority of nesting sea turtles. The ventral surface of foraging and unrestrained sea turtles was checked either by tilting the turtle or, at the time of lifting for release. Health assessments verified the individuals sampled were normal, healthy candidates for inclusion in the RI study. It should be noted that examination of nesting flatback turtles at night was limited by dark conditions, further complicated by restrictions on the use of light, particularly white light, due to its disturbance on the nesting turtles, so there was a risk that minor abnormalities were missed.

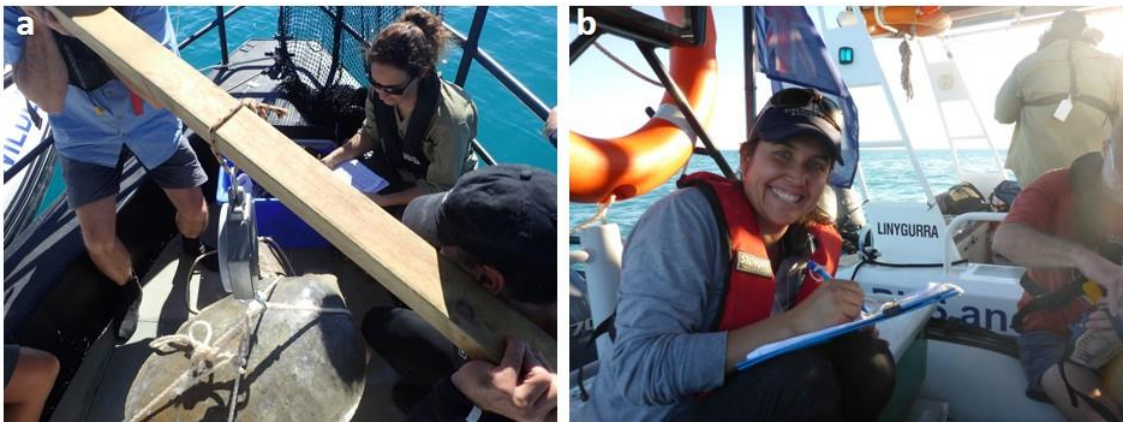

Figure 2.13 (a) Weighing a flatback turtle, and (b) Recording data in the field.

2.1.5 Blood samples

Every attempt was made to ensure blood was collected initially, prior to any additional disturbance or invasive procedure in order to ensure a consistent approach and avoid potential changes to blood values. All blood was collected and processed in a consistent manner to minimise variability between samples.

When sampling nesting flatback turtles on the TRD, all personnel at the front of the animal wore safety glasses (including the veterinarian collecting the blood samples). The wheelbarrow handles were tilted 60-70° bringing the sea turtle from a horizontal position into an inverted position (head down) which caused passive neck extension

as well as an increase in gravity dependent blood supply, facilitating good access and blood flow. With slight traction, causing further extension of the neck, the head was pressed toward the flat support. At all times the head was restrained from above to minimise risk of injury to the person holding the head. The dorsocervical sinus was used for blood collection (Owens and Ruiz 1980) by prepping the left or right dorsocervical sinus area with aqueous chlorhexidine gluconate/alcohol 5% (Chlorhex C ®, Jurox, Australia). Using a 1.5 in 18G needle attached to a 10-30 ml luer slip syringe (and occasionally an extension set was added for more active sea turtles), the needle was introduced at the level of the proximal third of the cervical region and lateral to the nuchal ligament, while applying negative pressure until a 'pop' indicated entry to the sinus. Ten to 30 ml of steadily drawn blood was collected, as if collected too slowly the blood would clot, while if collected too quickly it would haemolyse (Figure 2.14a and b). Once blood collection was completed (approximately one to two minutes in an inverted position), the flatback turtle was returned to a horizontal position) (Figure 2.10b).

This approach to blood collection was similar for sea turtles placed on a nally tub (Figure 2.14c). With the sea turtle's head over the side of the tub and neck extended, a person would assist to immobilise the head in position (not shown in photo) whilst blood was taken as previously described. However, for unrestrained sea turtles (majority of foraging flatback turtles) (Figure 2.14d), blood collection was slightly more challenging, and increased animal movement resulted in increased movement of the needle in the sinus, which had the potential to affect samples, for example increased haemolysis. For unrestrained nesting flatback turtles, the time directly after infilling the egg chamber and the start of covering up the nest, was the best time to attempt blood draw as the turtle had completed a crucial part of the nesting process and the neck was relaxed. Once she was further into the process of covering up the nest, the female's neck was tense and she would pull her head back towards her shell, making blood collection virtually impossible. Flatback turtles varied in their sensitivity to blood collection, some were needle shy and occasionally stopped the covering up process. For this reason, this approach was seldom used in nesting flatback turtles. While this

is a commonly reported way to collect blood from nesting sea turtles, further investigation into the best timing for unrestrained flatback turtle blood collection is required.

Figure 2.14 Flatback turtle blood collection techniques for (a, b) Nesting, and (c, d) Foraging turtles.

Due to issues associated with clotting and haemolysis during the collection of blood samples, primarily in the early stages of the research, rigorous blood handling techniques were developed and performed consistently to avoid artefacts. To prevent clotting, the needle was removed, and blood was transferred into anti-coagulant tubes at ambient temperature, as soon as possible post-collection. The blood was placed into the following tubes: 1 ml or 1.3 ml and 10 ml green topped tubes (MiniCollect® LH/Lithium Heparin, Greiner Bio-one, Austria; Lithium Heparin LH/1.3 screw cap SARSTEDT, Germany; Lithium Heparin BD Vacutainer®, BD-Plymouth, UK) and 1 ml

grey topped tube (Fluoride Oxalate [Fl-Ox], MiniCollect® Fx Sodium Fluoride/Potassium Oxalate, Greiner Bio-one, Austria) (see Table 2.1 for distribution details). The last 0.5 ml in the syringe was discarded to reduce the chance of introducing haemolysed cells into the tube.

Once blood was collected it was placed in a cold esky (portable cooler), ensuring blood tubes did not directly contact the ice bricks. If the option was available, samples were taken to the field station (laboratory converted accommodation) (Figure 2.15) and placed in the fridge throughout the sampling night. This maintained the samples at a constant temperature and avoided any detrimental effects from unnecessary handling.

Figure 2.15 Various field laboratory set ups.

If possible, the blood was centrifuged within six hours of collection, and the plasma separated to minimise any artefactual changes (Fudge 2000b). The 10 ml vacutainer tubes were centrifuged at 3500 rpm (or 1534 *g*) for 10 minutes (E8V LW Scientific Centrifuge, Lawerence, GA, USA), and the plasma was pipetted off into aliquots of

approximately 1 ml per 1.5 ml Eppendorf tube for various diagnostic tests and sometimes 1 ml of the leftover clot was also banked. The plasma was visually assessed for haemolysis and/or lipaemia. On the last two field trips, additional frozen blood samples were collected for heavy metal and toxicology testing (frozen plasma and whole blood) and whole blood was placed in 2.5% glutaraldehyde (1:1) for electron microscopy to identify haemoprotozoan parasites.

Multiple blood films were made using Lithium Heparin (Li-Hep) whole blood and a drop of Li-Hep blood was also placed on filter paper (Whatman™FTA™ [Flinders Technology Associates], GE Healthcare UK Ltd, Buckinghamshire, UK) and stored at room temperature for potential future molecular studies.

Samples were prepared for submission to Vetpath laboratory (Ascot, WA) (see Table 2.1 for details). The blood was carefully packaged in an esky, while the slides were fixed to the outside of the esky in a biological hazard bag along with the submission form and quotation. Due to the difficulties with temperature-induced blood changes, I-buttons (DS1921Z Thermochron, Thermodata® Pty Ltd, Eighty Mile Plains, Australia) were placed in the esky during transport to observe any temperature spikes. However, this was commenced after problems with sample storage had been addressed so few changes were observed.

Table 2.1 Sample details, tests and distribution.

Test	Sample	Volume/ Quantity	Storage conditions	Distribution/ Test location
Haematology	Li-Hep whole blood Li-Hep blood film	1 ml 1 slide	Refrigerated Room temperature	Vetpath
Biochemistry	Li-Hep plasma Fl-Ox whole blood	1 ml 0.25 ml	Refrigerated Refrigerated	Vetpath
Heavy metals	Li-Hep whole blood	1 ml	Frozen	Griffith University
Toxicology	Li-Hep whole blood Li-Hep plasma	1 ml 1 ml	Frozen Frozen	Griffith University
Various/future diagnostic testing	Li-Hep plasma Li-Hep whole blood Li-Hep clot Li-Hep blood film Li-Hep FTA Glutaraldehyde	3 ml 1 ml 1 ml 1-3 slides 1 drop 0.5 ml	Frozen Frozen Frozen Room temperature Room temperature Refrigerated	Murdoch University

Aside from the FTA card, additional blood films, glutaraldehyde samples, and samples for Vetpath, all samples were placed in the freezer (Table 2.1), however, prior to samples being banked some field-based laboratory testing was performed.

Due to the remoteness of the research, ensuring samples arrived at the laboratory to be analysed within 48 hours of collection was logistically challenging. For example, Eighty Mile Beach is 360 km from the Broome base and a courier depot, while Thevenard Island requires vessel transfers to the mainland town of Onslow with only road freight available in this town. This meant a significant amount of organisation was required to coordinate blood transportation from the study site to the lab and blood could only be collected on certain days to avoid exorbitant weekend courier rates i.e., blood could not be dropped off for courier transport on Friday, Saturday or Sunday. This meant that blood collection days were limited to Sunday through Wednesday, which was also influenced by transport times to the depot, and further limited by days people were travelling to depot location.

The samples in the esky were usually couriered to Vetpath, but occasionally these were taken as carry-on luggage by the author or a DBCA employee/volunteer travelling to Perth who could personally deliver the esky to Vetpath.

The remaining frozen samples were transported to Murdoch University, Perth in a portable fridge/freezer (Engel MT45FP 40L, Sawafuji Electric Co. Ltd, Japan) with frozen ice bricks, which was connected to a power source when there were delays between transfers and whenever power was available (e.g., on vessel from Thevenard Island to Onslow, Onslow airport) to minimise sample thawing.

Field-based tests

To determine the packed cell volume (PCV), a Li-Hep blood tube was inverted several times and duplicate plain glass capillary tubes (Statspin® Microhematocrit 40 mm untreated glass tubes, Iris, Chatsworth, CA, USA) were filled with whole blood and subsequently centrifuged at 12,000 rpm (6900 *g*) for three minutes using a ZipCombo Centrifuge (LW Scientific, Lawerence, GA, USA). The PCV was read immediately after

the cycle finished, otherwise the blood cells and plasma would mix and the results were inaccurate.

Total plasma solids (TPS) was calculated by placing a drop of the separated plasma on the refractometer window (Brix 0-32% Refractometer, LW Scientific, USA) and reading the TPS in g/L.

The i-STAT portable point-of-care analyser (Abaxis®, Abbott, Union City, CA, USA) was used in a subset of animals (n=87) based on the availability of the i-STAT analyser and cartridges. Once the animal's ID was recorded and the cartridge barcode was scanned, a drop of Li-Hep blood was placed into the cartridge well, taking care not to under or over-fill the cartridges. Within two minutes the analytes were calculated. The Chem8+ cartridge was used which measured blood urea nitrogen (BUN), creatinine, calcium, glucose, chloride, sodium, potassium, total carbon dioxide (tCO_2) and anion gap. The i-STAT required regular calibration and software updates (D. Bungey, REM Systems, personal communication, 1 December 2017). The CG4+ cartridges were also used in a small subset of animals from the Eighty Mile Beach flatback foraging trip (n=7). These turtles were processed on the liveaboard and time-sensitive analytes measured on the CG4+ could therefore be tested in the onboard laboratory within minutes from collection. Analytes measured on the CG4+ cartridges included pH, lactate, carbon dioxide partial pressure (pCO_2), oxygen partial pressure (pO_2), tCO_2, bicarbonate (HCO_3), base excess, and oxygen saturation (sO_2), some of which require temperature calibration. Due to the small sample size, the CG4+ values were not presented.

Commercial-based lab testing

Samples sent to Vetpath had haematology performed on the Cell-Dyn 3700 (Abbott Diagnostics, Germany), which was subsequently replaced with the Sysmex XN-1000 (Sysmex, Japan), so the last two foraging trip samples were analysed on this machine. Haematological parameters measured included haemoglobin (Hb), red blood cell count (RBCC), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), total white cell

count (TWCC) (automated and/or manual) and manual white blood cell (WBC) differential percentage and absolute counts. Automated TWCC were initially performed on the Cell-Dyn 3700 as according to the lab this machine could perform the count accurately on species with nucleated RBCs (Fudge 2000b, Vap et al. 2012, Han et al. 2016). However, when the machine was upgraded to the Sysmex XN-1000 automated TWCC could not be performed. It was then decided to review all blood films manually, including those of which automated TWCCs had been performed, to ensure consistency and due to the overwhelming evidence automated TWCCs are inaccurate in any species with nucleated RBCs such as reptiles, including sea turtles (Stacy and Innis 2017). The PCV was performed manually by placing Li-Hep blood in plain glass capillary tubes (Hurst Scientific, Australia) and centrifuging at 75 rpm for three minutes in the Haematokrit 20 (Hettich, Germany). Comments regarding cell morphology, platelet estimation, and any haemolysis were also included in the clinical pathology reports.

Plasma biochemical analytes were prioritised based on those considered the most useful for clinical diagnostics and those which were most comparable with other studies. Biochemistry was performed on the Beckman Coulter AU680 (Beckman Coulter, Japan), except for lactate dehydrogenase (LDH) which was sent to Western Diagnostics Pathology (Myaree, WA) for testing using an Advia Chemistry XPT (Siemens, USA). The biochemical panel included aspartate transaminase (AST), uric acid, glucose, total protein, calcium, and phosphorus, as these are considered to be the most useful analytes in reptilian diagnostics, as well as creatine kinase (CK), total bile acids, sodium, potassium, chloride, albumin, globulin, LDH, glutamate dehydrogenase (GLDH), which are also useful parameters (Wilkinson 2004, Campbell 2014, Eatwell et al. 2014). To facilitate comparisons with similar studies, analyses of alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin, BUN, cholesterol, triglyceride, magnesium, and iron were also performed (Whiting et al. 2007a, Whiting et al. 2007b, Flint et al. 2010b, Flint et al. 2010c, Guinea 2013, Whiting et al. 2014b, Stacy and Innis 2017).

Other diagnostic testing

The electron microscopy facilities (FEI Tecnai G^2 Transmission Electron Microscope, FEI Company, USA) at Department of Primary Industries and Regional Development (DPIRD) Diagnostic Laboratory Services (DLS) (South Perth, WA) were utilised to analyse blood samples in glutaraldehyde preservation for potential intraerythrocytic haemoparasites.

All spatial data and information pertaining to animal examination and biological sampling such as blood processing, in-field results, sample banking and distribution for testing were recorded using a Microsoft Excel datasheet (Microsoft Corporation, WA, USA).

2.1.6 Other data captured

A clinical examination form was developed to record pertinent information regarding each sea turtle sampled, with slightly different versions of the form used for the nesting and foraging sites (Appendix 3). The information collected included temporal and spatial data; signalment such as age-class, as well as other information pertaining to activity, morphometrics, and identification; environmental data; capture and restraint methods; sampling and survey techniques; findings from the basic physical examination and biological sampling (e.g., sample handling, processing, and results). This form constantly evolved over time and more data fields were added to improve data capture, but every effort was made to keep the original research dataset. Each sea turtle was given a unique 'WTR' ID. Sea turtles that underwent repeat sampling were also given a new WTR ID, which was linked to the original ID. Any additional sampling or procedures performed on animals as a component of other research were also captured on the form such as ultrasound, biopsy.

2.2 Morbidity and mortality investigations

Sea turtles stranding in WA between March 2016 and September 2018, provided an opportunistic source population for investigation of morbidity and mortality. Any sea turtle found dead, or which died or was euthanised in care, which was reported, in good condition, and accessible to the researcher was investigated as part of this study. Live sea turtles which were admitted to the Perth Zoo Veterinary Department (PZVD) (where turtles receive a complete systematic veterinary work up) were also included. The DBCA and the PZVD have a collaborative partnership to triage sick and injured sea turtles in WA. Data recorded on the WA Marine Turtle Stranding Form (Appendix 2), as well as those relating to clinical examination findings, sample collection, results of various diagnostic tests and in the case of dead sea turtles, necropsy examinations, were collated for analyses. While previous isolated investigations of clinical cases exist, this is the first state-wide, systematic longitudinal study to establish a point of reference for causes of stranding in WA. Specific details relating to the methods associated with sample collection from live and dead sea turtles have been provided in Section 2.2.

2.2.1 Disease investigations

Live sea turtles

On admission, live sea turtles were given a unique Perth Zoo wildlife identification number (i.e., R=reptile, XX=year, XX=month, X=nth admitted that year e.g., R1807288 was a reptile admitted in 2018 in July and was the 288th animal admitted in this year) and received an initial veterinary assessment including standard physical exam, weight, hydration status assessment, body condition scoring, fluorescein eye stain (BioGlo™ HUB Pharmaceuticals, Rancho Cucamonga, CA, USA), and cloacal temperature. The sea turtle's clinical record also included pertinent stranding information and there was a sea turtle admission form specially designed for turtles which had been housed in rehabilitation prior to admission to Perth Zoo (Appendix 4). All of the information was considered by the veterinarians when assessing individual

sea turtles, including initial diagnosis and prognosis, and when considering future diagnostic, treatment, and rehabilitation plans.

Generally, one to three days post-arrival at Perth Zoo, following the sea turtle having been warmed to its preferred body temperature (between 25°C to 30°C depending on the turtle's condition; at Perth Zoo turtles are maintained between 26°C to 28°C) and rehydrated, a standardised sea turtle diagnostic investigation was performed (Wyneken et al. 2006) (Figure 2.16). This included a thorough and more detailed clinical examination, four morphological measurements standardly taken as per the WA Marine Turtle Stranding Form (Appendix 2), and a range of diagnostic tests.

Figure 2.16 Part of the standardised sea turtle diagnostic investigation at Perth Zoo including (a) Radiographs, and (b) Blood collection.

Diagnostic tests included dorsoventral, lateral, and skyline radiographic views and if deemed necessary, contrast studies were undertaken, for example, in cases with suspected GI blockage using Barium Impregnated Polyethylene Spheres (BIPS, Medical I.D., Grand Rapids, MI, USA) or iohexol contrast (Omnipaque™, GE Healthcare, Australia) with serial imaging. Blood was collected from the dorsocervical sinus and cursory testing was performed in-house (including PCV, TPS, and glucose), and/or sent to Vetpath commercial laboratory for more in-depth analyses. For in-house PCV, whole blood in heparinised haematocrit tubes (Livingstone 75 mm Microhaematocrit capillary tube, Roseberry, Australia), and in duplicate, was centrifuged at 11,800 rpm (or 13,000 *g*) for five minutes (HaematoSpin 1300, Hawksley, Sussex, UK) and the PCV

measured. The TPS was subsequently determined by breaking the tube and placing a drop of plasma on the refractometer window (Brix 0-32% Refractometer, LW Scientific, USA) and reading TPS (g/L). Glucose was also introduced into the repertoire of basic in-house testing where a drop of whole blood was added to the glucometer (Accu-Chek® Active, Roche, Mannheim, Germany). Occasionally fresh in-house blood films were prepared in house and stained with Quick Dip (Amber Scientific, Traralgon, Victoria). Alternatively, whole blood in Li-Hep (MiniCollect® LH/Lithium Heparin, Greiner Bio-one, Austria) and two fresh blood films were submitted to Vetpath for a standard zoo reptile blood profile (ZR2), which consists of a complete blood count (CBC), including haematocrit and TWCCs and differentials at a minimum, and at other times additional haematological parameters (e.g., Hb, RBCs, MCHC, MCH, MCV), and 13 biochemical analytes including electrolytes (sodium, chloride, potassium), enzymes (AST, CK), proteins (total protein, albumin, globulin), minerals (calcium and phosphorus), plus metabolites (BUN, uric acid, and glucose). Haematology was performed on the Cell-Dyn 3700 (Abbott Diagnostics, Germany), which was subsequently exchanged for Sysmex XN-1000 (Sysmex, Japan) machine in April 2018, while all biochemistry was performed at Vetpath on the Beckman Coulter AU680 (Beckman Coulter, Japan) throughout the duration of the project.

The decision to submit to a commercial laboratory or analyse in-house was determined on a case-by-case basis at Perth Zoo, dictated by a variety of factors. Often in smaller sized animals there was insufficient blood volume to perform full laboratory testing so in-house testing was undertaken, likewise when high numbers of sea turtles were admitted it was not financially viable to send all samples for analysis to the commercial laboratory. However, in-house PCV, TPS, and glucose are all useful measurements for general health status (Norton et al. 2017), and can prove critical in correcting any life-threatening physiological imbalances, such as hypoglycaemic sea turtles, with blood glucose levels <2 mmol/L.

The results received from the ZR2 were compared against the Zoological Information Management System (ZIMS, Species360, Bloomington, MN, USA) RIs, however these

are wide RIs and aside from extreme values, most values fell within the interval or range. Furthermore, adequate sample size was only available for green turtle RIs on ZIMS, so cross species assumptions were made. Subsequently, relevant published RIs which were species, life stage (i.e., nesting or foraging) and age-appropriate were often consulted in conjunction with ZIMS RIs.

Following blood collection, samples collected and banked for future analyses included whole blood films, and a drop of blood on an FTA™ card stored at ambient temperature, while separated plasma, and occasionally clotted and whole blood were stored frozen.

Occasionally, if deemed necessary, other diagnostics including computed tomography (CT) scans and faecal floats were performed. The CT scans were performed by the diagnostic imaging specialist contracting to Murdoch University Animal Hospital (Dr Shona Reese, Vet Imaging Specialists) (Figure 2.17). While faecal samples were sent to the Parasitology Diagnostic Services at Murdoch University (Dr Amanda Ash, Paraquest).

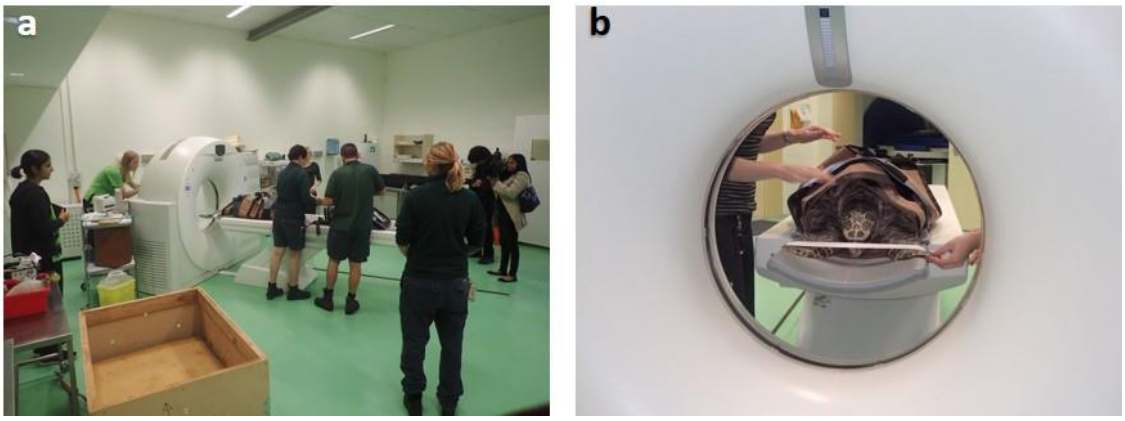

Figure 2.17 (a, b) CT scan of hawksbill turtle R1708111 ('Flo') at Murdoch University Animal Hospital.

Live sea turtles with suspicious external tumours (i.e., fibropapillomatosis) had a biopsy and histopathology performed (see Section 2.2.3).

Sea turtles rehabilitated at Perth Zoo received treatments (e.g., antibiotics, anthelmintic medication, and additional fluids), at the discretion of the attending veterinarian. Their progress during rehabilitation and response to treatment was closely monitored and the animals were often transferred off site to longer-term rehabilitation facilities once stabilised.

All information obtained throughout the sea turtle's time in care including clinical findings, test results, rehabilitation progress was recorded in the turtle's file on ZIMS.

2.2.2 Necropsies

Dead sea turtles

Sea turtles suitable for necropsy (n=75) had a decomposition score of one (D1) or two (D2), and occasionally in rare or unusual cases D3/D4 (Table 2.2; Figure 2.18), however, the cadavers in advanced state of decomposition seldom yielded diagnostic results. Fresh cadavers were preferred, however challenges obtaining such specimens (e.g., remoteness, limited resources) resulted in the majority of cadavers being frozen (51/75, 68.0%) with the remainder fresh (24/75, 32.0%) (see Table 2.2).

Table 2.2 Decomposition classification modified from Flint et al. (2009a) and Rowles et al. (2001).

Decomposition Score	Sample
D1	Live but subsequently died; euthanised. Necropsied fresh <48 hours.
D2	Dead, carcass in good condition. Fresh/edible. Necropsied fresh <72 hours or fresh frozen cadavers.
D3	Dead, carcass fair condition; decomposed but organs intact, early autolysis; OK to necropsy but usefulness of samples questionable.
D4	Dead, carcass poor condition; advanced decomposition with internal organs falling apart.
D5	Dead, mummified carcass with skin holding bones together.
D6	Dead, disarticulated bones with no soft tissue remaining.

Ideally, immediately post-mortem, cadavers were refrigerated to slow autolysis, however, if the necropsy was unable to be performed within 48 hours (max 72 hours) the specimen was frozen instead. Although, freezing causes cellular changes that

affect post-mortem results, namely cell rupture, it provides flexibility for scheduled necropsy and thawing out (which is a size dependent process taking between several hours to one week).

The majority of cadavers were transferred to Murdoch University Necropsy Suite for necropsy (55/75, 73.3%) (Figure 2.19), while the remainder (20/75, 26.7%) were performed in the field and at various locations (including Perth Zoo), to provide necropsy demonstrations to DBCA staff, veterinarians, nurses, and wildlife rehabilitators (Figure 2.20).

Figure 2.18 Examples of cadaver decomposition states including (a) D1, (b) D3, and (c) D5.

Figure 2.19 Post-mortem at Murdoch University Necropsy Suite.

Each sea turtle received a necropsy identification unique to the project ('WT' prefix, followed by the nth necropsy performed i.e., WT1 was the first necropsy). Necropsies

performed at Murdoch University were given an additional pathology number (year and n^{th} necropsy of corresponding year e.g., 18-250).

Prior to commencing the necropsy, a thorough history of the chronological details of the sea turtle was compiled including time of stranding and transportation, time in care (husbandry, veterinary assessment, ante-mortem diagnostics, treatment, rehabilitation) and death. Additionally, following the necropsy examination the start and end time of the examination was recorded on the dedicated necropsy datasheet developed for this project (Appendix 5).

A standardised approach for sea turtle necropsy examination was utilised to ensure the investigations were comprehensive and comparable to other studies (Flint et al. 2009a). In addition, non-standard methodologies specific to the project such as organ morphometrics, and grading barnacle burdens were included as potentially useful criteria. A necropsy equipment list and flow chart were developed to standardise and expedite the procedure, and for training purposes, and are detailed in Appendix 6 and Appendix 7, respectively.

Figure 2.20 Field necropsies showing (a) Necropsy demonstration on Thevenard Island, and (b) Weighing a large adult loggerhead turtle at Esperance DBCA compound.

The necropsy began with the recording of pertinent information pertaining to the signalment, morphometrics, scale nomenclature as well as any other identification (e.g., flipper tags) followed by a thorough external and internal examination of each

organ system, and the collection of a comprehensive set of samples (detailed below) (Figure 2.21).

Figure 2.21 Necropsy set up.

There were various reasons for recording specific morphometric measurements and data collection related to determination of species such as scale counts (namely costal and pre-frontal scales); age-class such as CCL, CCW, head width (HW); body condition such as standard depth (*SD*), SCL; sex such as *CT*, gonads; potential health status (body condition scoring, weight, external abnormalities, and epibiota coverage and growth); recent and previous food intake such as GI tract contents and overall gut fill percentage; and to establish baseline morphometrics for the major internal organs (e.g., heart, liver, spleen, brain, kidneys, adrenals, and occasionally gonads and thyroid). Furthermore, data were collected in relation to rare or data deficient areas, for example, lengths of the various intestinal segments and gastrointestinal contents were collected for flatback turtles given that such morphometric and dietary data are non-existent for this threatened sea turtle species.

During the systematic necropsy examination, any lesion and/or change to an organ system was recorded describing the distribution, size, colour, shape, consistency, and number.

Throughout the necropsy, digital photo recording was used to report a standard set of gross anatomical photos (Figure 2.22), including an array of head and body views,

plastron removed and organs in-situ, along with any internal or external anomalies (Figure 2.23).

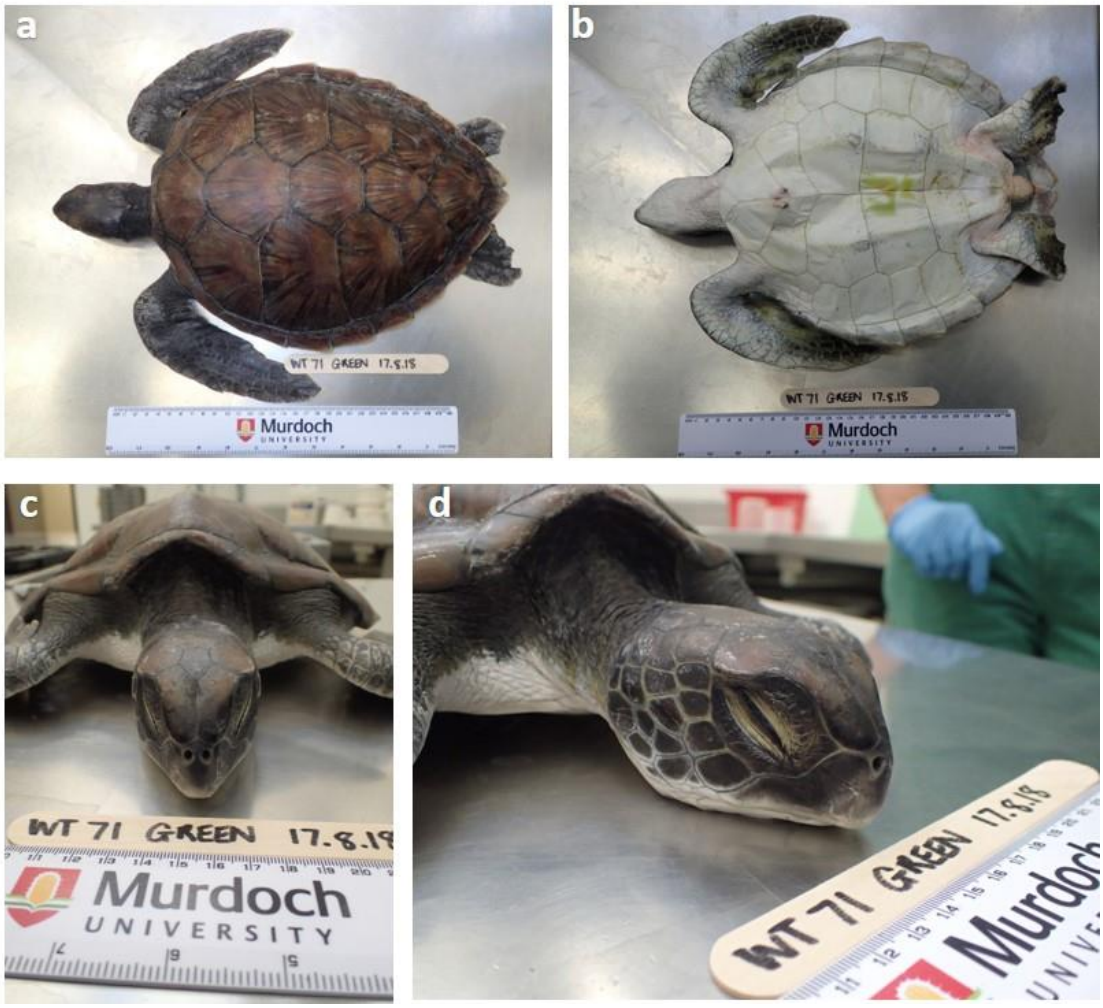

Figure 2.22 Selection of a standard set of photos taken during necropsy examination including (a) Dorsoventral body, (b) Ventrodorsal body, (c) Rostro-caudal head, and (d) Lateral head (photos taken bilaterally).

The necropsy procedure was performed as follows. With the sea turtle in dorsal recumbency, the coelomic cavity was opened by removing the plastron and making an incision at the skin/plastron junction and along the suture line between the ventral marginal scutes of the carapace and the inframarginal scutes of the plastron. Bone cutters/secateurs were sometimes required for four bony bridges at the cranial and caudal edges of the carapace/plastron bridge. With the sea turtle tilted away from the area of incision, so as to avoid perforation of bloated GI tract, starting cranially, caudal

traction was applied to the plastron, whilst removing the muscle attachments as close to the plastron as possible to enable assessment of pectoral muscle mass. The pectoral muscles were subsequently removed by making a circumferential incision around the forelimbs and removing attachments at the scapula-carapace junction. Once sufficient attachments were removed, the forelimb was rotated until it popped out. The pluck was removed by incising the hyoid bones to remove the tongue and glottis down to the distal GI tract, while the dorsally located organs (lungs and kidneys) were removed by cutting the fibrous connective tissue attachments close to the carapace and the epaxial muscles close to the spine. The brain was accessed by transverse incision caudal to eye (Figure 2.24 and Figure 2.25a), but occasionally additional incisions were made to remove the skull cap (Figure 2.25b) (Gordon 2005).

The extent of the necropsy examination and the set of samples collected was determined by the decomposition score and condition of the cadaver (fresh or frozen). For example a fresh D1 specimen with a complete necropsy examination performed in a laboratory had a thorough procedure including all biometrics, full set of samples and parasitology (see Section 2.2.4 below for further details), while a D4 sea turtle performed in the field had a modified necropsy examination with a minimum of four external measurements, a very brief internal examination and no samples collected aside from a flipper biopsy, collected for another research project. The sample collection techniques for submission are described below.

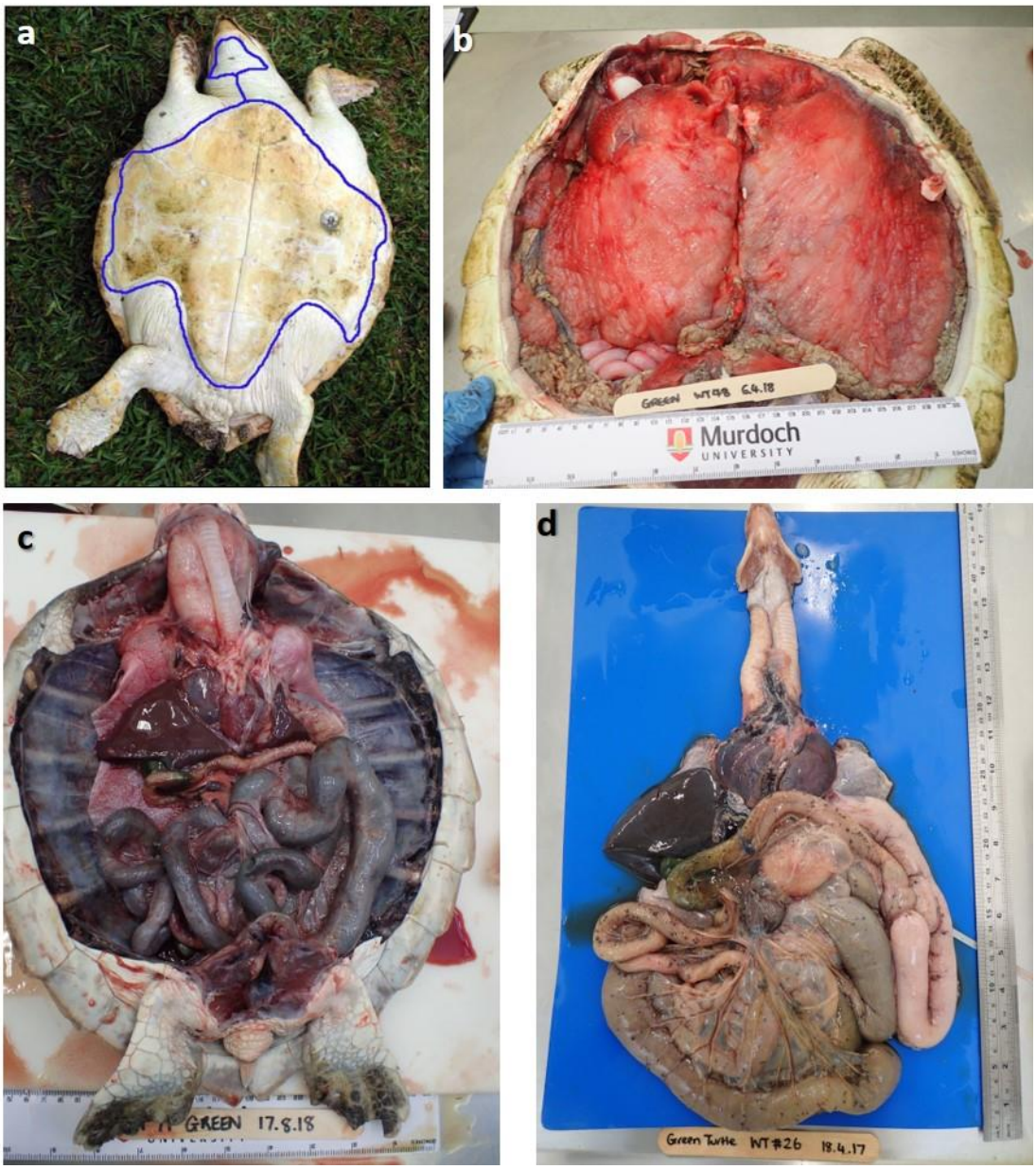

Figure 2.23 Different stages of the necropsy procedure showing (a) Incision outline (Wyneken 2001), (b) Plastron removed, (c) Organs in-situ forelimbs removed, and (d) Pluck removed.

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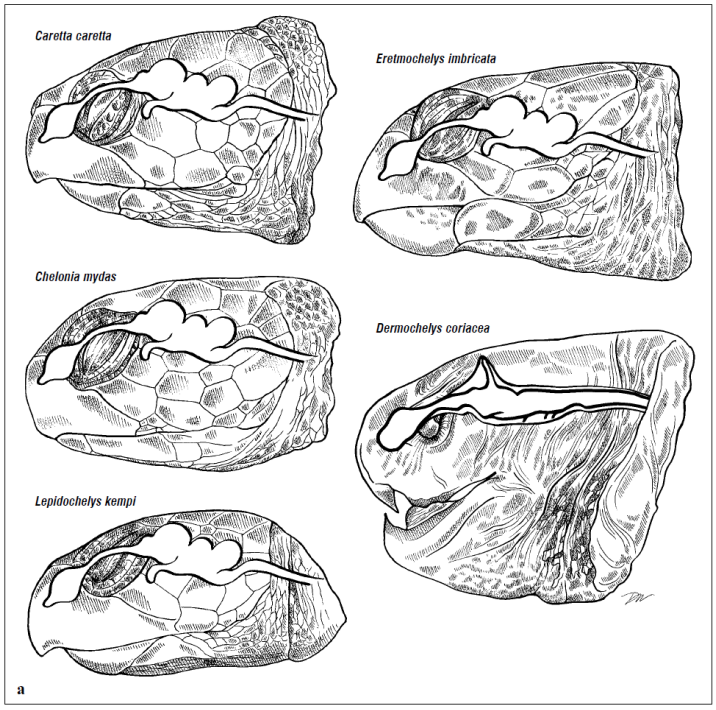


Figure 2.24 Brain locations for the various species (Wyneken 2001).

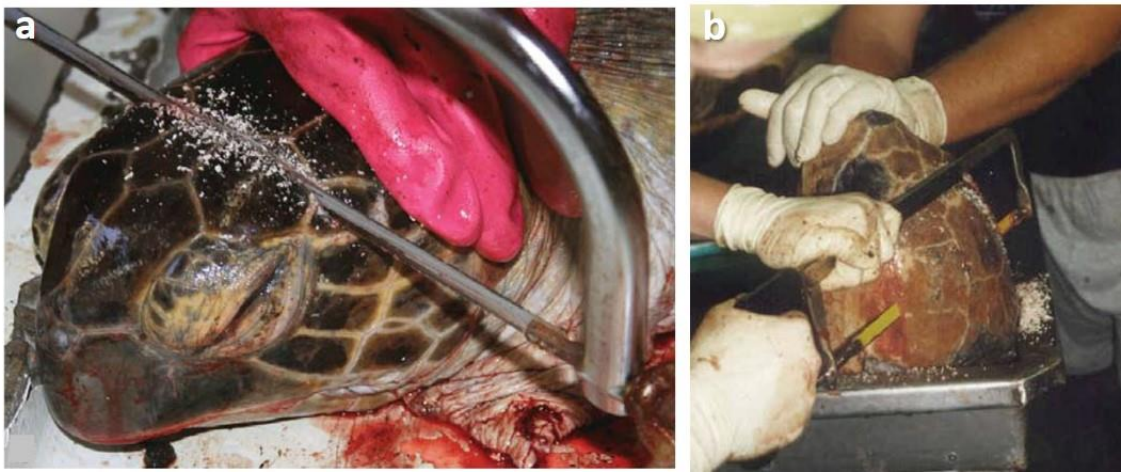


Figure 2.25 (a) First incision to remove brain (Flint et al. 2009a), and (b) Second incision if unable to access brain (Wyneken 2001).

Sample Collection

Histopathology

A standard set of tissues (<1 cm thick) were collected in 10% non-buffered formalin (NBF) (Formaldehyde Solution BP 34.5-38% w/w Pharmaust Manufacturing, Welshpool, WA) to be processed after necropsy. Samples collected for histopathology included the lung, heart, great vessels, spleen, liver, gall bladder, oesophagus, (crop), stomach, pancreas, multiple sections of intestine, thyroid, adrenal, gonad, kidney, urinary bladder, brain, spinal cord, and salt gland. When located, the sporadically present thymus was always processed. Additional samples collected but not routinely processed for histopathology included tongue/oral cavity, trachea, muscle, bone, fat, shell, skin, eye, and cloaca. Any abnormal lesions were also collected for histopathology, including fibropapilloma tumours which were collected from both live and dead sea turtles.

Microbiological culture

Microbiological culture was sometimes performed on cases with suspected microbial infections such as nodules or granuloma-type lesions to identify potential bacterial or fungal aetiological agents. However, this testing was only occasionally run as the majority of cadavers were frozen (68.0%) so culture was often unrewarding, and the testing was undertaken off-site at DPIRD so there were logistical and financial restraints. When microbiological sampling was undertaken, generally lung, liver or spleen were the organs that were tested. Using aseptic technique, Aimes swabs (Interpath, Melbourne, Australia) were utilised initially to collect samples for culture however, Stuart's media or charcoal swabs (Transwab Aimes charcoal, MWE Medical Wire, UK) were subsequently identified as preferred media for aquatics, so charcoal swabs were preferentially utilised from April 2017 onwards (Dr J.Bannister, DPIRD, personal communication, 20 April 2017). The swabs were kept in the fridge and transported in an esky to DPIRD, with most analyses being performed within 72 hours of collection (occasionally up to 96 hours when the timing coincided with long

weekends and public holidays). Subsequent to the mass mortality event in 2016 (Chapter five) screening was performed on samples using specific culture conditions to test for *Streptococcus iniae*. Serotyping was also performed on some isolates (e.g., *Salmonella* spp.).

General frozen tissue

A range of tissues were banked for future potential testing including lungs, heart, spleen, liver, kidneys, adrenals, skin, salt glands, brain, and any abnormal lesions. An aseptic swab collected into 10% PBS was the preferred technique for preservation of samples for molecular virology testing. However, this was infrequently collected.

Heavy metals

Tissues banked for potential heavy metal testing, were collected in plastic bags/vials and included 30 g (if possible) of liver, kidney, shell, skin, bone, muscle, and fat. Occasionally a nail sample and heart blood were also collected.

Toxicology

Tissues collected for future toxicology testing for Persistent Organic Pollutants (POPs) such as Polychlorinated Biphenyl (PCB), Dichlorodiphenyldichloroethylene (DDE), Polycyclic Aromatic Hydrocarbon (PAH) and Perfluorinated Alkaloids (PFAs), included 30 g (if possible) of liver, kidney, gonad, brain, muscle, and fat. Bile, gastrointestinal contents and occasionally heart blood were additionally banked with all tissues collected in glass jars/vials or aluminium foil.

Samples collected for other research projects

Samples were also collected for other collaborative research projects and included skin in 90% ethanol for stable isotope and population genetic studies; gastrointestinal contents for specific individuals where dietary preference was of interest such as all ages of flatback turtles; and the left humerus bone for skeletochronology studies, primarily for flatback turtles.

2.2.3 Histopathology

Samples collected for histopathology were processed by the Diagnostic Service at the Murdoch University Histology Laboratory (Figure 2.26). Standard techniques were used to process formalin-fixed tissue samples collected during necropsy, as well as external fibropapillomas/tumours for histopathology. Tissue samples were fixed for at least 48 hours prior to histopathological processing. Tissues trimmed to a thickness of 5 mm in labelled cassettes were processed (dehydrated with graded concentrations of ethanol, cleared with xylene and infiltrated with wax) in a Tissue-Tek VIP 5 Jr (Sakura, Japan) processor and embedded in paraffin using Leica EG1150H (Leica, Germany) embedder. The wax block was cut in 4 µm thick sections using Leica RM 2235 microtome (Leica, Germany) and slides were routinely stained with Haematoxylin and Eosin (H&E) and mounted with Dako Mounting Medium (Dako, Glostrup, Denmark) to Hurst glass slides (Forrestdale, Australia) for histopathological analysis.

Figure 2.26 Tissues sections of fibropapilloma tumours for histopathological processing.

The standard set of aforementioned post-mortem tissues were stained with H&E for the majority of sea turtles selected (namely D1 or D2) for histopathological analysis. Any tissue lesions collected were also processed initially. Subsequent differential stains were selectively performed at the discretion of the attending veterinary pathologist, as an adjunct for diagnosis. Based on microscopic characteristics, differential stains were used for various purposes such as to identify infectious microorganisms, tissues, deposits, and pigments.

Additional differential stains

Special stains utilised for specific diagnostic purposes are described below:

- Gram Twort: differentiates gram positive (purple) and negative (red) bacteria in suspected bacterial infections (Swisher 2002).
- Congo Red: has an affinity for amyloid used when amyloid deposits suspected which stain bright red (Vowles and Francis 2002).
- Perl's Prussian Blue: confirms the presence of haemosiderin (iron-complex) particularly in melanomacrophages, which stain bright blue when positive (Churukian 2002).
- Melanin Bleach: a negative stain for presence of melanin in pigment (Churukian 2002).
- Modified Trichrome: used for various diagnostic testing and identifies microorganisms such as microsporidia which stain pinkish-red if positive (Jones 2002).
- Martius, Scarlet and Blue Trichrome: identifies mature collagen in fibrosis which stain blue (Jones 2002).
- Giemsa (Microwave): classically used to demonstrate haemoparasites in peripheral blood films, but also used to identify microorganisms such as microsporidia which stain dark-blue (Swisher 2002).
- Ziehl-Neelsen (ZN): detects acid-fast organisms (mainly mycobacteria) which stain bright red (Swisher 2002).
- Von Kossa: identifies calcium deposits where a positive reaction stains calcium metallic black (Callis 2002).
- Periodic Acid-Schiff (PAS): identifies presence of fungi which stain purple-magenta if positive (Totty 2002).
- Periodic Acid-Ammoniacal Silver (PAAS): also identifies presence of fungi which stain black (Churukian 2002).
- Alizarin Red: used as a differentiation stain where calcium crystals stain red-orange but urate does not (Churukian 2002).
- Lithium carbonate extraction-hexamine silver: demonstrates urate crystals which stain black (Totty 2002).
- Ammoniacal silver: used for urate crystals which also stain black (Churukian 2002).
- Pizzolato: used to identify calcium oxalate crystals which stain black (Luna 1968).
- Calcofluor White: used with fluorescence microscopy to detect organisms such as microsporidia which fluoresce when positive (Harrington and Hageage 2003).

An immunohistochemical (IHC) stain for Toxoplasma was performed at the Murdoch University Histology Diagnostic Laboratory using Thermoscientific Polyclonal (Goat) Antibodies CN: PA5-16921 (Thermoscientific, Rockford, IL, USA).

Neospora, Cryptosporidia, *Batrachchochytrium dendrobatidis* (Chytridomycosis), and a repeat Toxoplasma IHC were performed at DPIRD. The antibodies were produced in-house but were all finished using the Envision dual-link K4063 antibodies (Dako, Denmark), with a DAB substrate (Dako, Denmark) and all samples generally had Protease K (Dako, Denmark) treatment for five minutes at 37°C prior to being incubated overnight at 4°C.

A murexidine colour change test was utilised for differentiating urate (purple) and calcium (colourless) crystals (Casimire-Etzioni et al. 2004).

Additional steps for non-standard tissue

Shell and bone were not routinely processed except as indicated such as in gross pathology cases (e.g., FP of the shell, gout/pseudogout), when elected by the attending veterinary pathologist, or when determining if bone or shell "trauma" occurred pre- or post-mortem. Bone processing required a longer fixation of four to five days in formalin followed by decalcification in 5% nitric acid (Univar, Auckland, New Zealand), usually overnight. The decalcification process was monitored every 12 hours until it was sufficiently soft to cut with a Feather trimming blade no.130 (Feather Safety Razor Co, Japan).

Shell is composed of bone with a thick overlying keratin layer. For processing, shell was placed in 10% KOH (Chem Supply, Port Adelaide, SA) to soften the keratin followed by 5% nitric acid to decalcify the bone (Gerard Spoelstra, Murdoch University, personal communication, October 2017). The timing for each was dependent on the sample and would be checked every 12 hours and if the tissue (keratin or bone) was softening in their respective targeted chemicals, the sample was removed and subsequently processed.

Digital recording

Photomicrographs were taken of interesting or unusual cases using a DP22 Olympus camera (Olympus, Tokyo, Japan) mounted on an Olympus BX50 light microscope (Olympus, Tokyo, Japan) using an image acquisition system (DP-SAL, Olympus, Tokyo, Japan).

2.2.4 Parasitology

Blood and other parasites observed grossly during necropsy or recovered from specialised parasitological techniques (organ washes) were collected in either 10% NBF for morphological identification or 90% ethanol (Ethanol Absolute 100, Scot Scientific, Welshpool, WA) for molecular identification.

Organ washes

Organ washes were performed on a subset of sea turtles (25/75, 33.3%) based on time constraints and turtle condition, and provided a qualitative assessment for parasite presence or absence and type but were by no means quantitative. Organ washes were principally performed on coelomic cavity fluid; heart/great vessels; lung/trachea; spleen; liver; kidneys; brain; and salt glands to detect spirorchiids. Additional organ washes were occasionally performed on gall bladder; urinary bladder; oesophagus/crop; small intestine; large intestine; mesentery; adrenals; gonads; and pancreas to detect other parasites and infrequently spirorchiids.

The wash involved placing the organ in premixed chilled saltwater (8 g/L), sliced in such a way as to open vessels. The organ was vigorously mixed with the saltwater then removed. The remaining liquid was then left to settle so the parasites would sink to the bottom. After approximately 20 minutes the supernatant was decanted and the remaining sediment examined under a dissecting microscope. Any flukes were pipetted out and kept preserved in either 10% NBF or 90% ethanol. Spirorchiids were preferentially saved in formalin, particularly those from fresh cadavers which were intact and suitable for morphological identification. Gastrointestinal flukes are

considered very difficult to morphologically identify, consequentially, these were preferentially placed in ethanol for molecular identification (Adjunct Prof. D. Blair, James Cook University, personal communication, 9 July 2016) (Figure 2.27).

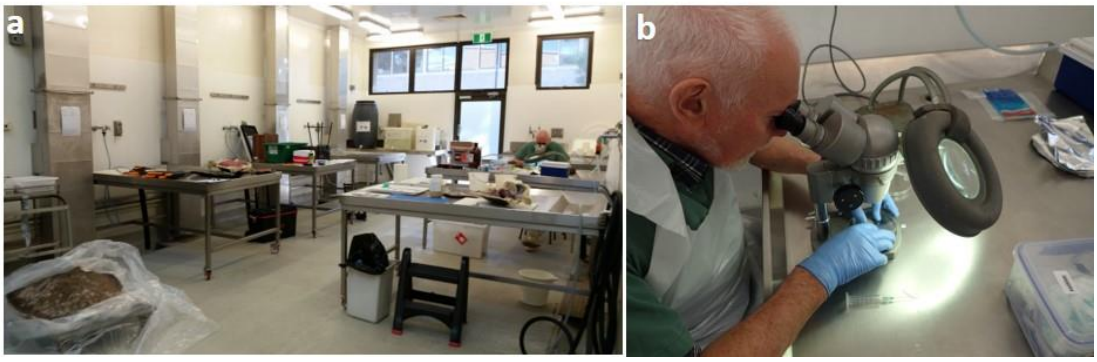


Figure 2.27 (a, b) Multiple necropsies were undertaken during a training week in parasitological techniques with Prof. David Blair.

Morphological identification

Spirorchiids, other flukes, cestodes, and nematodes either grossly observed or retrieved from organ washes were placed in 10% NBF and processed (by the researcher) in the Murdoch University Parasitology Museum. Once fixed, parasites were washed several times in distilled water to remove the fixative, then placed in undiluted Gower's carmine stain (Gower 1939) overnight, or until the parasites appeared very darkly staining. The parasites were rinsed in distilled water again, then destained by adding 0.5% HCl (Scharlau, Barcelona, Spain) to distilled water until the stain was removed from the parenchyma i.e., parasite was pale pink, and the gonads were reddish, or when no more stain was removed during the acid change. The parasites were then passed through an alcohol series (70%, 90%, 100%, 100%) for 30 minutes each. Then the parasites were cleared in methyl salicylate (Sigma Aldrich, St Louis, MO; China), and mounted in Canada Balsam (Sigma-Aldrich, St Louis, MO; Spain). Photographs were taken of the mounted parasites using a dissecting microscope (Olympus SZX7, Tokyo, Japan) with a mounted camera (Olympus DP27, Tokyo, Japan) using imaging software (Olympus cellSens Standard, Tokyo, Japan) and

a specialist sea turtle parasitologist was consulted for species identification (Adjunct Prof. D. Blair, James Cook University).

Unstained nematodes fixed in formalin were identified via a parasitology electronic key developed by DPIRD in 2018 with the assistance of a specialised nematode parasitologist (J. Mitchell, DPIRD).

Metacestodes cysts were also identified morphologically by specialised cestode parasitologist (Emeritus Prof. I. Beveridge, University of Melbourne)

Molecular Identification

Helminths (trematodes, cestodes, and nematodes) collected in 90% ethanol for the molecular identification component were processed at DPRID-DLS, Molecular Division.

DNA extraction

The Qiagen QIAmp DNA Mini Kit (DNeasy blood and tissue kit, Qiagen, Germany) was used for DNA extraction, as per the manufacturer's instructions. The sample was lysed by adding Buffer ATL and proteinase K, vortexing and incubating. Buffer AL was added, vortexed and incubated, then ethanol was added and vortexed. The mixture was placed in a special spin column and processed through a series of washes and centrifuging (Heraeus™ Pico™ 17 Centrifuge, Thermofisher Scientific, USA). Finally, an elution buffer was added, the sample incubated and centrifuged and the elution becomes the DNA extraction. This was kept frozen until further processing for up to three months.

Conventional trematode 28S PCR protocol

A primary master mix was prepared including RNA free water, Qiagen Hotstar Taq Master Mix, LSU5 (10 µM) forward primer and 1500R (10 µM) reverse primer and added to 2 µL of DNA template. A conventional PCR was run on Thermal Cycler (FHL Eppendorf, Hamburg, Germany) and using the trematode 28S PCR protocol i.e., denaturation 15 minutes @ 95°C for one cycle; PCR denaturation, annealing and

extending cycle for one minute @ 95°C, 45 seconds @ 52°C each for 40 cycles; followed lastly by the extending stage 1.5 minutes @ 72°C for 40 cycles; then left at 4°C for [Infinity] (Littlewood et al. 2000).

QIAquick® PCR Purification Kit (Qiagen, Germany) was used to prepare the spirorchiid eggs in frozen tissue for PCR.

PureLink™ Quick Gel Extraction Kit (Invitrogen Thermo Fisher Scientific, Vilnius Lithuania) was used to extract DNA when multiple bands arise (band stab or band cut out in gel).

Gel electrophoresis

A 3% agarose gel was made by heating 3 g of Ultrapure™ Agarose powder (Invitrogen, Life Technologies, Carlsbad 92008, CA, USA) and 100 ml TAE buffer (2 M Tris-Acetate, 0.05 M EDTA, pH 8.3) (Eppendorf AG, Hamburg, Germany) for two minutes and 30 seconds or until the agar completely dissolved. With both the teeth strip (small for 10 wells, large for 20 wells) and tray locked into the levelled agar plate holder, once cooled, the gel was poured into the tray, then placed in the fridge for approximately 20 minutes until the gel set.

A piece of Parafilm® (paraffin film) was placed on a microtube rack and pressed in a way to create small wells, same number as number of samples for electrophoresis, plus one or two ladders at the end(s). The gel was run at 90 V for 60 minutes (Bio-Rad PowerPac® Basic, Hercules, CA, USA). The gel was subsequently read using a UV light, and a photo was taken. Consistent bands were cut from PCR gels using sterile scalpel blades. Purified PCR product was sent for Sanger sequencing (PD+) at the Australian Genome Research Facility (AGRF, Melbourne, Victoria) and Geneious software (version 11.1.5, Biomatters, Ltd, Auckland, NZ) was used to clean up the sequences and run Basic Local Alignment Search Tool (BLAST) analysis. Species were identified by phylogenetic analysis with the assistance of a specialist molecular parasitologist at DPIRD (Dr T. Miller, DPIRD).

2.2.5 Additional diagnostic testing

Electron microscopy

The electron microscopy facilities (FEI Tecnai G^2 Transmission Electron Microscope, FEI Company, USA) at DPIRD-DLS were utilised for a single case to analyse the muscle pseudocysts in *C. mydas.* Formalin-fixed tissue was used, however, glutaraldehyde is the preferred preservative for this diagnostic technique.

Molecular diagnostics

Next generation sequencing was performed in the Western Australian State Agricultural Biotechnology Centre laboratory at Murdoch University under the supervision of Dr M. O'Dea (Veterinary virologist) and was used to screen a subset of fresh-frozen fibropapilloma tumours, potentially connected to ChHV5, and also a potential muscle parasite of unknown aetiology (Figure 2.28).

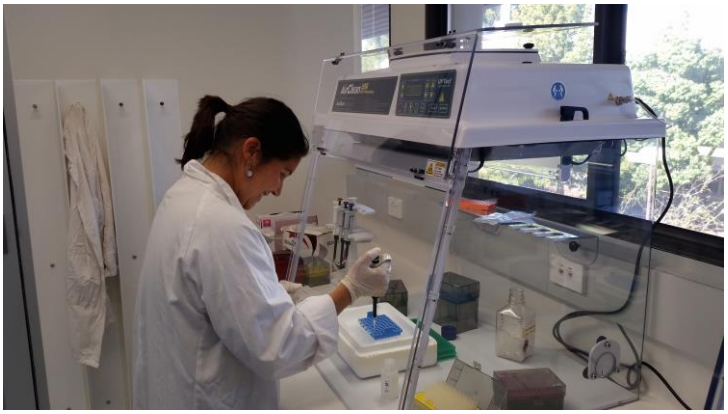


Figure 2.28 Next Generation Sequencing at Murdoch University.

All extractions for next generation sequencing and PCR were performed using Invitrogen Isolate II Genomic DNA Extraction Kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) as per manufacturer's instructions using the technique described by O'Dea et al. (2016), with the exception that the RNAse treatment step was omitted.

For next generation sequencing, amplification of Klenow reaction involved the preparation of AmpliTaq Gold 360 Master Mix (Life Technologies, Carlsbad, CA, USA)

including buffer, Primer NGS1 (CCTTGAAGGCGGACTGTGAG) and water, which was added to 2.5 µL Klenow reaction template. Conventional PCR was run on SimpliAmp™ Thermal Cycler (ThermoFisher Scientific, USA) using a standard protocol i.e., denaturation five minutes @ 95°C for one cycle; two PCR cycles including one minute @ 95°C, 56°C and 72°C each for five cycles and 20 seconds @ 95°C, 30 seconds @ 55°C and one minute @ 72°C for 35 cycles; and finally the extending step seven minutes @ 72°C and left on hold @ 14°C.

The primers used for conventional PCR on the fibropapilloma tumours were the same at both Murdoch University and DPIRD-DLS (Ehlers et al. 1999), while the primers created by Rector et al. (2005) were used to test for papillomavirus (AR-E1F2 and AR-E1R4 and 9).

Products were electrophoresed in a 1.5% agarose gel containing gel red stain for one hour at 90V. Bands of the expected size (215-235 bp) were excised (Ehlers et al. 1999) and purified using Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA) using the standard protocol for DNA purification by centrifugation.

Library preparation and barcoding was performed using the Nextera XT DNA library preparation kit (Illumina) following the manufacturer's instructions but with minor modifications as per O'Dea et al. (2016) and final sequencing was performed on an Illumina MiSeq using a V3 2x300 Flowcell.

Read data were *de novo* assembly and performed using SPAdes (Centre for Algorithmic Biotechnology, St Petersburg, Russia). Contigs were then searched for homology to viral agents against the NCBI nr protein database using DIAMOND v0.7.1 with an e value cut-off of 10^-5 (O'Dea et al. 2016).

Conventional PCRs were run on muscle to test for microsporidia and apicomplexan at Murdoch University in Dr Tim Hyndman's Reptile Pathogen Laboratory using V1-PMP2 primer pair (Muller et al. 1999) and pan-Apicomplexan primers 1135F and 1503R (Garner et al. 2006) for the respective microorganisms.

CHAPTER 3

Developing a health baseline for flatback turtles (*Natator depressus*) in Western Australia

3.1 Introduction

The flatback turtle is a specially protected threatened species with vulnerable status at both state and federal levels, but classified as 'data deficient' internationally (Commonwealth of Australia 2017b, IUCN 2019). Some of the largest flatback turtle foraging and nesting stocks are found in WA (Department of the Environment and Energy 2019f). Flatback turtles face a number of anthropogenic threats including large scale industrial and coastal development, and climate change. Any related issues associated with such threatening processes, such as chemical pollution, degraded habitat, changes in diet and environmental stress may be revealed in haematological and blood chemistry values, and changes to values over time.

Blood RVs are the blood analyte values from a reference individual. Reference individuals are clinically healthy individuals identified through health assessments of wild populations. The RVs are used to calculate blood RIs which typically encompass the middle 95% (between 2.5^{th}-97.5^{th} percentiles) of a healthy reference population and are central to any baseline health survey (Deem et al. 2001, Geffré et al. 2009). Once established, these RIs can be used to assess the health status of the individual or population, identify clinically unhealthy individuals, monitor trends, and serve as prognostic indicators (e.g., when treating sea turtles at rehabilitation centres).

While RIs may provide an indication of systemic health in any species, development of RIs in wild sea turtle populations is more challenging. As ectotherms, the influence of intrinsic (e.g., species, sex, age/size, and physiologic status) and extrinsic factors (e.g., season, habitat, epibiota load, capture method, and other environmental conditions) on blood parameters requires careful consideration when assessing RVs. Variation in blood collection, sample handling, and processing techniques can also have significant impacts on RVs (Campbell 2014), and comparisons between different RIs should be undertaken with caution (Stacy and Innis 2017). Furthermore, chelonians are reported

to have greater variation in plasma biochemistry values between individuals in a species, due to physiological regulation (Sposato et al. 2002).

Further challenges associated with the development of RIs in sea turtles include potential uncertainties with the case definition of a 'clinically healthy' individual, the analytical methodology, and the selection of analytes. For example, convenience sampling typically used in wildlife studies by selecting animals of unknown health can introduce error. Field methods can also introduce error, for example, through the use of manual techniques which may lack the same level of precision as well as the quality assurance and quality control of an accredited laboratory, potentially affecting the RIs (Flatland et al. 2010, Friedrichs et al. 2011). In a study by Flint et al. (2010b), only a small proportion of haematological and biochemical values were significantly different for unhealthy and healthy loggerheads, suggesting the possibility that not all unhealthy loggerheads had been excluded from the reference group, which is not surprising due to the subtlety of clinical signs in reptiles. The possibility that disease may not be reflected by haematological or biochemical changes is another potential explanation for difficulties in defining a 'clinically healthy' individual. Reference intervals are typically based on analytes relevant to humans or domestic animals which may or may not be applicable to reptiles (Herbst and Jacobson 2003), with many analytes lacking sensitivity and specificity in this group (Stacy and Innis 2017). Additionally, certain analytical methods are not validated (Campbell 2014), such as the traditional bromocresol method for albumin measurement, with electrophoresis far superior in accurately quantifying plasma protein fractions.

While numerous RIs have been developed for sea turtles, inappropriate statistical analyses associated with inadequate sample sizes, unreported confidence intervals and out-dated outlier detection methods, may result in inaccurate RIs (Flint et al. 2010b). Following the International Federation of Clinical Chemistry (IFCC) and Clinical Laboratory and Standards Institute (CLSI) guidelines, adopted by the American Association of Veterinary Clinical Pathology (ASVCP), and using advanced statistical

techniques to estimate RIs can address these issues and result in narrower RIs with improved accuracy (Friedrichs et al. 2011).

The only turtle RIs available in WA are for loggerhead turtles from Ningaloo and Dirk Hartog Island (Trocini 2013). There is one other report with RVs for foraging green turtles from Montgomery Reef in WA (Guinea 2013). There are two published RV papers and one report from off-shore islands and reefs for green turtle populations (Whiting et al. 2007a, Guinea 2013, Whiting et al. 2014a), and a published RV paper for hawksbill turtles from off-shore islands (Whiting et al. 2014a), all from Commonwealth waters in the east Indian Ocean. Other baseline blood parameter studies in Australia include RIs for hawksbill turtles from the Northern Territory (NT) (Whiting et al. 2014b), RIs for loggerhead and green turtles in Queensland (Flint et al. 2010b, Flint et al. 2010c), and RVs for green turtles in the NT (Hamann et al. 2006, Whiting et al. 2007a). The two Queensland studies further examine the frequently lacking investigations of relationships between clinically unhealthy individuals and RIs (March et al. 2018). Only four previous flatback turtle bloodwork studies exist including three studies in nesting turtles and one study in hatchlings. The nesting flatback studies include blood indicators for stress-related handling techniques on Bare Sand Island, NT (Guinea 2016), blood respiratory physiology research on Curtis Island and Mon Repos in Queensland (Sperling et al. 2007), and more recently the relationship between microbiome and blood values on Crab Island, Queensland and Port Hedland, WA (Scheelings et al. 2020), while the hatchling study investigated blood indicators and anaerobic metabolism at Mon Repos (Pereira et al. 2013).

Despite a significant increase in flatback turtle research, particularly in ecology and biology fields, major knowledge gaps remain in relation to foraging flatback turtles. Furthermore, there are no published disease-related studies on flatback turtles and only one published study related to health in this species (Scheelings et al. 2020). Reference interval health research in Australian sea turtles has primarily focused on biochemistry, due to the logistical challenges associated with remote fieldwork, sample collection, and transportation for laboratory analysis required for

haematological analysis. While WBC values are occasionally reported (Flint et al. 2010b, Flint et al. 2010c, Trocini 2013, Scheelings et al. 2020), PCV is the only commonly recorded haematological parameter. A physiological research paper also reported Hb in nesting flatbacks (Sperling et al. 2007), while another study measured trace metals in nesting flatback turtles (Ikonomopoulou et al. 2011). Only three of the RI/RV studies investigated nesting turtles including one loggerhead (Trocini 2013) and two flatback turtle surveys (Guinea 2016, Scheelings et al. 2020); the remaining studies involved foraging turtles. Few of these studies have more than 120 animals (Flint et al. 2010b, Flint et al. 2010c, Trocini 2013) and not all studies follow the CLSI-IFCC recommendations to determine RIs making comparisons between studies challenging.

The flatback turtle is currently facing pressure from high levels of industrial development, along with other mounting environmental pressures impacting species globally. As a sentinel species of environmental health, development of baseline values is imperative to monitor the impact of pressures for ongoing management (Aguirre and Lutz 2004, Deem and Harris 2017). Therefore, the aim of this study was to aid the establishment of haematological and biochemistry RIs for flatback turtles. The objectives were to:

(i) Use the RVs from flatback turtles at several nesting sites in WA to develop RIs.

(ii) Determine if there are differences in RVs in nesting and foraging turtles which support the development of individual RIs for these groups.

(iii) Investigate the level of agreement between point-of-care field tests and laboratory values and determine if delay of processing and laboratory analysis affected the analyte values.

3.2 Methods

3.2.1 Study sites and animals

The health baseline research was conducted at three study sites between 2016-2019 over multiple trips (n=9 field trips; n=45 sampling days). The field sites comprised medium density rookeries at Thevenard Island (21.4563°S, 115.0021°E) and Eighty Mile Beach (19.5931°S, 121.2694°E), and resident foraging grounds at Roebuck Bay (18.0585°S, 122.2831°E) and Eighty Mile Beach. These foraging grounds include flatback turtles of mixed genetic stocks, while the rookeries are distinct genetic stocks (Dr S. Whiting, DBCA, personal communication, 17 February 2020). Nesting sites were chosen for ease of access and to sample rookeries across a latitudinal range. The foraging sites were selected because these are the only two known locations to obtain these samples. The study sites varied from undeveloped (Eighty Mile Beach), industrialised (Thevenard Island) to urbanised (Roebuck Bay) sites. Study animals included flatback turtles nesting at Thevenard Island (n=72) and Eighty Mile Beach (n=74), and turtles foraging in Roebuck Bay (n=42) and in waters adjacent to Eighty Mile Beach (n=7). Nesting turtles were sampled at night during the nesting season, during the warmer, wetter months (November-February), while foraging turtles were sampled during daylight hours in the dry season, predominantly winter (May-August).

3.2.2 Capture and restraint

Nesting flatback turtles selected for sampling were hand-caught returning to the ocean, mostly post-nesting, and restrained on a purpose-built TRD or 'turtle barrow' (Figure 3.1). Very occasionally blood was collected without the TRD, directly post-oviposition, however this sometimes resulted in the turtle abandoning the final nesting stage (covering up), and accordingly, this approach was seldom used. While the TRD potentially caused some stress, it minimised interruption of the nesting process, was quicker, improved human and animal safety, and aided examination of the turtle. Foraging turtles were captured by either rodeo (Limpus and Walter 1980)

or dip net from DBCA vessels during dedicated foraging flatback surveys and restrained by hand for blood sampling.

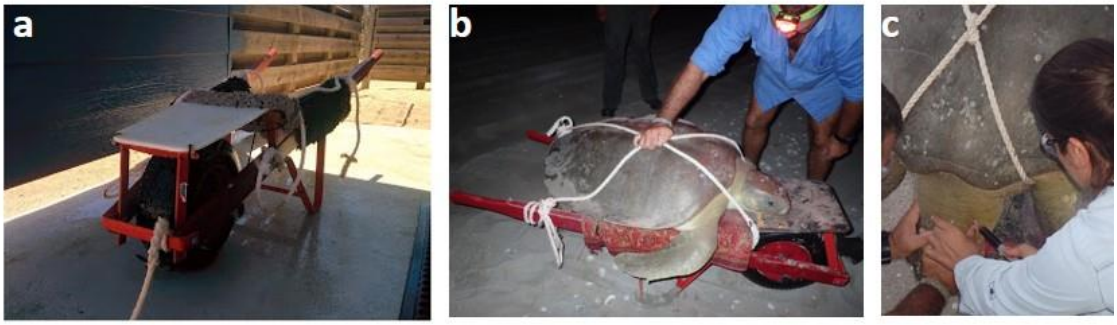


Figure 3.1 (a) Turtle restraining device (TRD), otherwise known as the 'turtle barrow', (b) Nesting flatback turtle restrained on TRD, and (c) Blood collection from dorsocervical sinus.

3.2.3 Health assessment and blood sampling

Every attempt was made to consistently collect blood immediately after capture and prior to any additional disturbance or invasive procedure to minimise the potential impact on blood values. Blood samples were usually collected within three minutes of capture but occasionally this could extend up to 20 minutes. A basic external physical examination was conducted including body condition scoring i.e., based on a scale of one to five, where one is emaciated and five is obese (Norton and Wyneken 2015) and recording of any abnormalities (e.g., flipper amputations, neurological deficits and presence of barnacles). Morphometrics (to the nearest 0.1 cm) were recorded including CCL and CCW using a flexible measuring tape. Foraging turtles were weighed using a mechanical scale (to nearest 0.5 kg) and males also had various tail measurements taken (not reported). Thorough examination was limited for nesting turtles sampled at night, further complicated by restrictions on light usage to minimise disturbance (Department of Parks and Wildlife 2013b).

The dorsocervical sinus area was prepared with aqueous chlorhexidine gluconate/alcohol 5% (Chlorhex C®, Jurox, Australia) and using a 1.5 in 18G needle, 20-30 ml of blood (or a maximum of 3ml/kg) was collected (Figure 3.1c) and immediately

transferred into anti-coagulant tubes then placed in a cooler (see Table 3.1 for distribution details).

Table 3.1 Sample details, tests and distribution.

Test	Sample	Volume/ Quantity	Storage conditions	Distribution/ Test location
Haematology	Li-Hep whole blood Li-Hep blood film	1 ml 1 slide	Refrigerated Room temperature	Vetpath
Biochemistry	Li-Hep plasma Fl-Ox whole blood	1 ml 0.25 ml	Refrigerated Refrigerated	Vetpath
Heavy metals	Li-Hep whole blood	1 ml	Frozen	Griffith University
Toxicology	Li-Hep whole blood Li-Hep plasma	1 ml 1 ml	Frozen Frozen	Griffith University
Various/future diagnostic testing	Li-Hep plasma Li-Hep whole blood Li-Hep clot Li-Hep blood film Li-Hep FTA Glutaraldehyde	3 ml 1 ml 1 ml 1-3 slides 1 drop 0.5 ml	Frozen Frozen Frozen Room temperature Room temperature Refrigerated	Murdoch University

The 10 ml vacutainer tubes were centrifuged at 3500 rpm (or 1534 *g*) for 10 minutes (E8V LW Scientific Centrifuge, Lawerence, GA, USA), and the plasma pipetted off into aliquots. Multiple blood films were prepared using Li-Hep whole blood. Samples were prepared for submission to Vetpath laboratory (Ascot, WA) (Figure 3.2, Table 3.1).

3.2.4 Field-based tests

The PCV was determined using duplicate plain glass capillary tubes (Statspin® Microhematocrit 40 mm untreated glass tubes, Iris, Chatsworth, CA, USA) filled with whole Li-Hep blood and centrifuged at 12,000 rpm (6900 *g*) for three minutes using a ZipCombo Centrifuge (LW Scientific, Lawerence, GA, USA). While TPS were determined using a refractometer (Brix 0-32% Refractometer, LW Scientific, USA).

The i-STAT portable point-of-care analyser (Abaxis ®, Abbott, Union City, California) was used for a subset of animals (n=87) using Li-Hep whole blood and a Chem8+ cartridge to measure sodium, potassium, chloride, glucose, and BUN, as well as creatinine, ionised calcium, tCO_2, anion gap, haematocrit (HCT) and Hb. As

temperature was not recorded, temperature-dependent ionised calcium and tCO_2 could not be used. Only the most useful analytes (sodium, potassium, chloride, glucose, BUN) were reported. Although haematological parameters measured on these analysers are considered unreliable (Stacy and Innis 2017), HCT was also reported to facilitate comparison with the other PCV values. Cases with a BUN i-STAT reading of '<1 mmol/L' (n=54) were converted to 0.5 mmol/L.

Figure 3.2 Field laboratory set-up on foraging turtle survey aboard Department of Biodiversity, Conservation and Attractions patrol vessel, Worndoom, Eighty Mile Beach Marine Park.

3.2.5 Laboratory testing

Haematology was performed on the Cell-Dyn 3700 (Abbott Diagnostics, Germany) or Sysmex XN-1000 (Sysmex, Japan) (Appendix 8) and included Hb and RBCC. The PCV was determined manually using Li-Hep blood in 75 mm plain glass capillary tubes (Hurst Scientific, Australia) and centrifuging at 7500 rpm for three minutes in the Haematokrit 20 (Hettich, Germany). The MCHC, MCH, and MCV values were subsequently calculated. Estimated TWCCs were performed by counting WBCs in 10 representative fields under 10x or 40x and multiplying the WBC count by the square of the objective. The differential percentage was performed by counting 100 WBCs

(identifying as heterophil, lymphocyte, monocyte/azurophil, eosinophil or basophil) under 40x and the absolute count calculated from the percentage of the TWCC (Heatley and Russell 2019b). The heterophil: lymphocyte (H: L) ratio was also calculated as a potential indicator of stress. Automated TWCCs were performed initially, however due to inaccuracies with automated TWCCs in species with nucleated RBCs, only manual counts were utilised. Comments regarding cell morphology, haemoparasites, platelet estimation, and haemolysis were also reported. Generally, the grading scheme was mild (1+, 1-5 cells), moderate (2+, 6-10 cells) and severe (3+, >10) when describing numbers of abnormal cells per 100x objective (1000 times magnification) or degree of change, for example polychromasia, anisocytosis. All blood film examinations were performed by board-certified clinical pathologists at VetPath laboratory, a NATA accredited laboratory.

Plasma biochemical analytes were prioritised by those considered the most useful for clinical diagnostics and were most comparable with other studies. Biochemistry was performed on the Beckman Coulter AU680 (Beckman Coulter, Japan) (Appendix 8), except for LDH which was tested using an Advia Chemistry XPT (Siemens, USA) at Western Diagnostic Pathology (Myaree, WA). The biochemical panel included AST, uric acid, Li-Hep glucose (nesting turtles only), Fl-Ox glucose, total protein, calcium, phosphorus and calcium: phosphorus (Ca: P) ratio as these are considered to be the most useful analytes in reptilian diagnostics, as well as CK, ALP, BUN, cholesterol, triglyceride, sodium, potassium, chloride, magnesium, iron, LDH, GLDH, which are also useful parameters (Wilkinson 2004, Campbell 2014, Eatwell et al. 2014, Stacy and Innis 2017). To facilitate comparisons with similar studies, we also analysed ALT and total bilirubin. However, these analytes are considered of little clinical significance in reptiles/sea turtles because biliverdin, not bilirubin is the final breakdown product of haemoglobin in reptiles, while ALT has particularly wide tissue distribution (Stacy and Innis 2017) so provides little diagnostic value. Albumin (and globulin calculation) were reported even though methods used are considered less accurate (Macrelli et al. 2013) when compared to electrophoresis. Bile acids were also measured as they have been

reported as useful (Eatwell et al. 2014). See Appendix 8 for laboratory measurement method details.

3.2.6 Data analysis

All spatial and temporal data, as well as information pertaining to animal examination and biological sampling including blood processing, in-field results, sample banking and distribution for testing were recorded on a datasheet and transcribed into a Microsoft Excel spreadsheet (Microsoft Corporation, Washington, USA).

Exclusion criteria

Exclusion criteria were established to remove any individuals or values identified as unsuitable in the development of RIs (Table 3.2). Classification of flatback turtles as immature was based on morphometrics less than the smallest nesting flatback turtles in this study (i.e., <73.0 cm CCL, <63.0 cm CCW and <50.0 kg) and these were removed. Duplicates (n=4) were excluded except if sampling re-occurred more than three months apart. Clinically sick individuals were excluded based on diminished body condition (i.e., body condition score of 3/5 or less), fresh or active injuries, or infections detected during examination, usually supported by clinical pathology. As the purpose of this research was to develop the first RIs for flatback turtles, individuals with atypical blood values (outliers) without visible abnormalities were not excluded at this stage.

Due to the remoteness of the research, ensuring samples arrived at the laboratory to be analysed within the recommended 48 hours of collection was logistically challenging. Eighty Mile Beach is 360 km from the town of Broome and a courier depot, while Thevenard Island requires vessel transfer to the mainland town of Onslow where only road freight is available. Further, Eighty Mile Beach and Thevenard Island study sites are respectively 1700 km and 1400 km from the laboratory in Perth. The cut-off point for time to laboratory analysis was therefore increased from 48 hours to 60 hours. Likewise, the time to decanting and i-STAT analysis was increased from the recommended six hours from sampling time to eight hours for similar logistical

reasons. Statistical testing was performed to ensure these extended times did not significantly impact blood values.

Development of RIs and outlier detection techniques

Based on the results from the preliminary testing (Appendix 9), RIs and/or descriptive statistics were calculated separately for nesting and foraging turtles using Reference Value Advisor (RefVal) (Solberg 1995). RefVal v2.1 (National Veterinary School of Toulouse, 2009) is a set of macroinstructions for use in the Microsoft Excel program which calculates mean, median, standard deviation (SD), minimum, maximum and runs normality (Anderson-Darling), symmetry, and outlier detection tests (Tukey and Dixon-Reed) (National Veterinary School of Toulouse 2012). Regardless of the distribution, RIs were computed using five different methods including parametric, robust, non-parametric, the former two with and without transformation (Box-Cox) (Geffré et al. 2011). The confidence intervals (CIs) were calculated using non-parametric tables (>120) (TPS) or non-parametric bootstrapping (<120), except for standard parametric CIs, where parametric bootstrapping was used (Friedrichs et al. 2011, Geffré et al. 2011). The RIs were colour-coded to denote values in accordance with the CLSI guidelines or presence of outliers or non-parametric distribution.

A combination of graphical (histograms and boxplots) and statistical tests were used to assess distribution, check normality (Anderson-Darling test) and calculate outliers (Tukey and Dixon-Reed tests) for the two datasets using RefVal program and R (R Core Team 2019). Tukey outliers outside the interquartile fences were reported in RefVal as suspect

$$Q1 - 1.5 * IQR \quad (1)$$

or

$$Q3 + 1.5 * IQR \quad (2)$$

or extreme

$$Q1 - 3 * IQR \quad (3)$$

or

$$Q3 + 3 * IQR \quad (4)$$

where IQR = Interquartile Range and Q1 = 25th percentile and Q3 = 75th percentile. Dixon-Reed outliers were calculated using the formula D/R > 0.3, where D is the difference between the most extreme value and next closest value, and R is the range of all values. Outliers reported for each analyte followed CLSI guidelines in RefVal and were dependent on RI calculation method (i.e., with or without transformation) (Appendix 10 and 11). Each outlier was scrutinised and retained (especially Tukey outliers prone to over-detection when compared to Dixon outliers) unless there was a genuine reason for exclusion. If an individual had three or more outliers, deemed to be of clinical significance, all analytes for that individual were removed. Additional criteria used in outlier decision-making included removal of extreme Dixon outliers (except i-STAT) and either removal of all extreme outliers or removal of extreme and suspect outliers if the mean with and without the outliers differed by more than 20% (except for ALP for nesting turtles, as well as absolute and differential basophil counts for foraging turtles, further explained in Section 3.4 – Discussion). Subsequently, extreme outliers tended to be removed except those deemed to be of lesser clinical significance (e.g., BUN maintained over a wide-range), or if i-STAT values were extreme yet correlated with their comparative laboratory values (likely due to inherent inaccuracies of the portable blood analyser). Due to a lack of flatback turtle RIs, outliers comparable to other sea turtle RVs (e.g., ALP and basophils) were preferentially retained.

Table 3.2 Global exclusion criteria showing which analytes were removed for individuals deemed unsuitable for inclusion (removed prior to outlier analysis).

Criteria	Number excluded[a]	Comment
Immature	3	All analytes removed for juveniles.
Sick individuals	5	All analytes removed for sick/unhealthy individuals.
Duplicates	3	Duplicates[b] excluded had all analytes removed.
Haemolysed samples	15 (18)	Removed laboratory haematology (red cell parameters) and biochemistry for laboratory-reported haemolysis. Samples with slight haemolysis had only laboratory haematology removed (n=3).
Blood film issue	32 (38)	Non-Li-Hep blood films excluded. Blood films with degenerated cells excluded. Differential % reported for blood films with clumping only (n=6).
>60 hours to laboratory analysis	35 (38)	All laboratory haematology (red and white cell parameters) and biochemistry removed except for three cases only biochemistry removed (due to timing differences for the different machines).
>8 hours to decant from sampling time	14	All laboratory biochemistry as well as in-house TPS removed.
>8 hours to i-STAT analysis	9	All i-STAT values removed.
>20% difference for PCVs[c]	18	All laboratory haematology (RBC parameters) including in-house PCV removed.
Other miscellaneous laboratory/machine/sample issues	9	All analytes removed if sample was lipaemic. RBC parameters removed for clotted whole blood. Abnormal i-STAT results removed.

[a] Total number of individuals affected by exclusion criteria, noting some overlap among the various exclusion criteria.

[b] Duplicates were individuals with resampling less than three months apart. For these individuals, the first sample was retained unless it did not meet exclusion criteria, in which case the second sample was selected. The single duplicate for which resampling was more than three months apart was also excluded, due to haemolysis of one sample.

[c] PCV values were removed for individuals where PCV difference i.e., (larger PCV – smaller PCV) / smaller PCV *100, was more than 20%.

While non-parametric RI calculation methods are recommended for sample size >120, the majority of the analyte sample sizes in this study were <120 (Friedrichs et al. 2011). The RI calculation method selected was based on which was the narrowest, whilst ensuring it followed CLSI guidelines, and involved preferentially selecting robust methodology with this method representing the best estimation of underlying distribution for symmetrical data with sample sizes <120 (Horn et al. 1998, Friedrichs et al. 2012).

Boundary conditions, health indicators and temporal associations

Once outliers were removed and the final nesting and foraging RVs were determined, we examined statistical differences for morphometrics and analytes between various boundary conditions including life stage, rookeries, foraging males and females, and season. Potential health indicators such as polychromasia (increased RBCs with basophilic cytoplasm indicative of RBC regeneration), anisocytosis (variably sized RBCs), presence and severity of RBC vacuolation/novel haemoparasites (vacuoles may be related to the presence of a haemoparasite), as well as basophil presence, were also examined by reintroducing outliers and most exclusion criteria (i.e., the whole dataset excluding duplicates), including criteria affecting haematological parameters (as this did not affect identification of these anomalies). We also investigated potentially stressful scenarios such as nesting time-period, time restraint, and number of sampling events by examining temporal effects on particular analytes used to indicate stress (e.g., H: L). Student's t-test (after Levene's test for equal variances with Welch modification for unequal variances) or Mann-Whitney U test (with continuity correction if necessary) were performed following Anderson-Darling goodness-of-fit test to assess distribution on parametric or non-parametric data, respectively. For parameters with more than two groups, ANOVA or Kruskal-Wallis test for corresponding parametric or non-parametric data were run (followed by post-hoc Tukey's HSD or Dunn test with Bonferroni adjustment respectively).

We examined temporal relationships between analytes and processing, as well as any statistical significance between body size and analyte values (by reintroducing

exclusion criteria related to time, processing, and maturity for the respective testing) using Pearson's correlation of the coefficient for linear data otherwise Spearman's rank correlation was utilised. Correlations were ranked as per previously established values i.e., excellent (0·93 to 0·99), good (0·80 to 0·92), fair (0·59 to 0·79) or poor (<0·59) (Bauer and Moritz 2008).

Comparisons between point-of-care and laboratory values

Bland-Altman plots were used to analyse agreement of the combined dataset of the final nesting and foraging RVs and examine the proportional bias for analytes with multiple measurement methods (PCV, total protein/TPS, sodium, potassium, chloride, glucose, BUN), plotting the difference against the mean. As Bland-Altman requires parametric distribution of the difference data, Passing-Bablok regression analysis (along with Pearson's correlation and Cusum linearity test) was used as an alternative for non-parametric data. Statistical significance was set at a level of $p \leq 0.05$.

3.3 Results

3.3.1 Size and age-class

Descriptive statistics for morphometrics are provided for nesting, as well as mature and immature foraging flatback turtles (Table 3.3).

Nesting flatback turtles were longer with a median CCL of 89.2 cm (n=129, range: 76.9-95.0 cm) than foragers CCL of 87.4 cm (n=45, range: 79.8-92.8 cm) and wider with a CCW of 75.0 cm (n=130, range: 67.1-79.9 cm) than foragers CCW of 71.0 cm (n=45, range: 63.4-77.0 cm) (excluding juveniles). Nesting turtles at Thevenard Island rookery were wider with a median CCW of 75.7 cm (n=66, range: 67.1-79.9 cm) than those at Eighty Mile Beach CCW of 74.0 cm (n=64, range: 69.0-78.8 cm) but were not statistically different lengths (W=2000, p=0.05). Mature female foraging turtles were significantly wider with a mean CCW of 71.9 cm +/- 2.28 (n=26, range: 68.3-77.0 cm) than mature male foragers CCW of 69.3 cm +/- 3.16 (n=19, range: 63.4-73.8 cm) and

had heavier body weights 79.2 kg +/- 5.87 (n=21, range: 70.5-93.5 kg) than their male counterparts at 72.4 kg +/- 9.98 (n=18, 56.5-94.5 cm), but similar lengths (t(40)=2, p=0.08).

Table 3.3 Descriptive statistics for clinically healthy flatback turtle morphometrics.

Measurement	Boundary group	Units	n	Mean/ RV[a]	Median	SD	Min	Max
CCL[b]	Nesting	cm	129	89.2	89.2	2.89	76.9	95.0
	Foraging (mature)		45	86.9	87.4	3.09	79.8	92.8
	Foraging (immature)		3	63.6	61.4	8.42	56.5	72.9
CCW[c]	Nesting	cm	130	74.7	75.0	2.46	67.1	79.9
	Foraging (mature)		45	70.8	71.0	2.95	63.4	77.0
	Foraging (immature)		3	56.4	56.2	5.05	51.4	61.5
Weight	Nesting	kg	1	84.0				
	Foraging (mature)		39	76.1	75.5	8.64	56.5	94.5
	Foraging (immature)		3	32.3	28.5	12.31	22.3	46.0

[a] Single reference value for n=1; [b] Curved carapace length; [c] Curved carapace width.

Due to the low number of juveniles sampled (n=3), no statistical testing could be performed using maturity and correspondingly, as most of the sample population were adults, there were no significant correlations between size and any analytes. See Appendix 12 and 13 for juvenile flatback turtle blood results.

3.3.2 Reference intervals and outliers

For nesting flatback turtles, there were 144 outliers in 35/51 analytes (Appendix 10). Outlier evaluation resulted in removal of a total of 72 outlier values (i.e., outliers met exclusion criteria). This included removal of all analytes for five individuals due to either questionable health, sample quality or more than three outliers. White blood cell parameters for four individuals, and i-STAT results from one individual were also removed due to sample or test issues. This resulted in the removal of a total of 37 outliers for nesting turtles.

For foraging flatback turtles, there were 49 outliers in 24/50 analytes (Appendix 11) and after careful inspection, 18 outlier values were removed. This included three

individuals which had all analytes removed for questionable health status, sample quality issues, or if the individual had three or more clinically significant outliers; this resulted in a total of 12 outliers being removed.

For the remaining nesting (n=35) and foraging (n=6) statistical outliers not mentioned above, extreme values were removed for nesting turtles except for ALP, MCHC, BUN, GLDH and i-STAT sodium and BUN (see methodology for outlier decision-making process but predominantly if the mean did not change significantly i.e., >20%). All extreme outliers were excluded for foraging flatback turtles, except for the basophil differential extreme outliers. Once the appropriate outliers were eliminated, the final RIs/RVs for nesting and foraging flatback turtles were calculated.

Initial analysis, after global exclusion criteria were applied but before outlier analysis, found 38/51 analytes (or calculated parameters) were statistically different between foragers and nesters (Appendix 9). Accordingly, separate nesting and foraging RIs and/or descriptive statistics including distribution type and method of RI calculation as well as final number of outliers (post-outlier removal) are presented in Table 3.4a and 3.4b, and Table 3.5a and 3.5b**.**

Although the majority of the blood analyte sample sizes in this study were <120 and non-parametric RI calculation methods are preferentially used for sample sizes >120, non-parametric methods were selected for analyte sample sizes <120 to meet CLSI guidelines i.e., other RI calculation methods violated the guidelines. Only three juvenile turtles were sampled but one individual did not meet inclusion criteria (of <8 hours to decant from sampling time) further reducing sample size for biochemistry and in-house TPS (n=2) (see Appendix 12 and 13 for juvenile RVs).

Table 3.4a Laboratory haematological and biochemical RIs and descriptive statistics for nesting flatback turtles.

Analytes	SI Units	n	Mean[a]	Median	SD	Min	Max	Lower limit (90% CI)	Upper limit (90% CI)	Distrib-ution[b]	Method[c]	Number outliers[d]
PCV	L/L	63	35.1	34	5.3	27	50	27.6 (27.0 - 28.6)	48.8 (43.6 - 50.0)	NG	NP	0
Hb	g/L	63	109.9	109	13.6	82	141	86.2 (82.0 - 92.1)	140.4 (130.0 - 141.0)	NG	NP	0
RBC	10^{12}/L	62	0.25	0.2	0.09	0.1	0.5	0.10 (0.10 - 0.12)	0.48 (0.40 - 0.50)	NG	NP	0
MCHC	g/L	63	315.4	324	31.4	204	350	260.9 (244.1 - 262.6)	386.4 (367.2 - 403.6)	NG (S)	R	3/2
MCH	pg	59	470.2	462	149.1	242	1010	244.8 (242.0 - 279.8)	980.0 (729.4 - 1010.0)	NG	NP	0
MCV	fL	61	1446.7	1445	444.1	750	3818	779.1 (710.2 - 872.1)	3194.0 (2764.4 - 3681.9)	NG (S)	RT	0
WBC	10^9/L	80	7.43	7.44	2.33	2.56	14.6	2.86 (2.30 - 3.44)	14.14 (12.85 - 15.33)	G (S)	RT	0
Heterophils	%	84	55.2	55	16.8	24	91	21.70 (17.0 - 26.6)	88.8 (83.6 - 93.7)	G	P	0
Heterophils	10^9/L	80	4.23	4.40	1.86	1.30	8.40	1.43 (1.30 - 1.70)	8.31 (7.72 - 8.40)	NG	NP	0
Lymphocytes	%	84	26.8	27	14.6	1	57	2.0 (1.0 - 3.1)	54.9 (50.6 - 57.0)	G*	NP	0
Lymphocytes	10^9/L	80	2.10	2.18	1.37	0.03	5.10	0.13 (0.03 - 0.19)	4.84 (4.61 - 5.10)	NG	NP	0
Monocytes	%	84	5.0	4	3.6	0	17	0.0 (0.0 - 0.1)	13.0 (11.0 - 17.0)	NG	NP	0
Monocytes	10^9/L	80	0.40	0.32	0.32	0.00	1.50	0.00 (0.00 - 0.00)	1.39 (0.88 - 1.50)	NG	NP	0
Eosinophils	%	84	13.0	13	6.4	0	30	0.0 (0.0 - 2.0)	27.8 (22.9 - 30.0)	G*	NP	0
Eosinophils	10^9/L	80	0.96	0.92	0.58	0.00	2.65	0.00 (0.00 - 0.12)	2.47 (2.03 - 2.65)	G*	NP	0
Basophils	%	82	0.0		0.0	0.00	0.00	0.0 (0.0 - 0.0)	0.0 (0.0 - 0.0)	na	na	
Basophils	10^9/L	78	0.00		0.00	0.00	0.00	0.00 (0.00 - 0.00)	0.00 (0.00 - 0.00)	na	na	
H: L ratio		71	2.21	1.68	1.78	0.44	8.63	0.51 (0.44 - 0.62)	8.61 (5.87 - 8.63)	NG	NP	0
CK	U/L	84	743.6	457	631.9	107	2580	125.8 (107.0 - 172.1)	2375.3 (2010.3 - 2580.0)	NG	NP	0
AST	U/L	85	180.2	179	82.9	80	337	84.0 (75.4 - 94.0)	318.5 (294.6 - 340.8)	G (S)	RT	0
ALT	U/L	83	6.2	6	3.3	1	19	1.1 (1.0 - 2.1)	17.0 (12.9 - 19.0)	NG	NP	0
ALP	U/L	82	51	53	7.9	32	132	34.2 (32.7 - 36.2)	98.7 (87.2 - 114.3)	NG (G)	T	0
Bilirubin T	µmol/L	86	3.08	2.4	1.96	1.0	9.5	1.00 (1.00 - 1.00)	9.31 (6.08 - 9.54)	NG	NP	0
BUN	mmol/L	86	1.65	1.7	2.64	0.5	7.6	0.53 (0.47 - 0.60)	5.30 (4.43 - 6.28)	NG (G)	T	0/1
Bile acids	µmol/L	84	1.5	1	0.7	0	5	1.0 (0.0 - 1.0)	3.0 (2.0 - 5.0)	NG	NP	0
Uric acid	mmol/L	86	0.064	0.063	0.016	0.031	0.112	0.038 (0.031 - 0.041)	0.107 (0.096 - 0.112)	NG	NP	0
Glucose Li-Hep	mmol/L	48	4.56	4.7	0.72	3.1	6.4	3.09 (2.82 - 3.37)	6.03 (5.73 - 6.32)	G	P	0/1
Glucose Fl-Ox	mmol/L	38	3.75	3.8	0.91	2.0	5.4	1.88 (1.49 - 2.29)	5.62 (5.19 - 6.04)	G	P	0
Cholesterol	mmol/L	86	6.83	6.8	3.58	3.0	17.5	3.42 (3.09 - 3.75)	13.51 (12.08 - 14.99)	NG (S)	RT	0/1
Triglyceride	mmol/L	86	7.07	7.1	4.40	1.4	18.8	1.41 (1.04 - 2.03)	16.42 (14.85 - 18.02)	G (S)	RT	0

Analytes	SI Units	n	Mean[a]	Median	SD	Min	Max	Lower limit (90% CI)	Upper limit (90% CI)	Distrib-ution[b]	Method[c]	Number outliers[d]
Sodium	mmol/L	86	153.0	153	3.3	145	161	145.2 (145.0 - 148.0)	159.0 (158.0 - 161.0)	NG	NP	0
Potassium	mmol/L	86	4.68	4.7	0.45	3.5	5.8	3.77 (3.64 - 3.90)	5.59 (5.45 - 5.72)	G	P	0/1
Chloride	mmol/L	86	111.8	111	5.0	100	121	103.0 (100.0 - 104.0)	121.0 (119.0 - 121.0)	NG	NP	0
Total protein	g/L	86	45.6	47	6.8	31	62	31.9 (30.0 - 33.9)	59.3 (57.2 - 61.3)	G	P	0
Albumin	g/L	86	13.9	14	2.5	9	19	9.2 (9.0 - 10.0)	18.0 (18.0 - 19.0)	NG	NP	0
Globulin	g/L	86	31.7	32	4.7	22	44	22.3 (21.0 - 23.7)	41.1 (39.7 - 42.5)	G	P	0
Calcium	mmol/L	86	3.87	3.71	1.03	2.09	7.33	2.28 (2.09 - 2.57)	6.02 (5.88 - 7.33)	NG	NP	0
Phosphorus	mmol/L	86	3.51	3.52	0.80	1.55	5.09	1.91 (1.69 - 2.14)	5.11 (4.86 - 5.34)	G	P	0/1
Ca: P ratio		86	1.09	1.08	1.21	0.71	1.76	0.77 (0.73 - 0.81)	1.65 (1.53 - 1.79)	NG (G)	T	0
Magnesium	mmol/L	85	4.22	4.24	0.67	2.80	6.22	2.89 (2.70 - 3.08)	5.56 (5.35 - 5.76)	G	P	0/2
Iron	µmol/L	86	7.45	8.0	2.87	2.0	14.4	3.00 (2.00 - 3.18)	13.83 (12.41 - 14.40)	NG	NP	0
LDH	U/L	86	572.4	527	225.0	259	1214	283.8 (259.0 - 328.2)	1203.5 (1065.4 - 1214.0)	NG	NP	0
GLDH[e]	U/L	27	36.16	22	36.39	1.3	151	1.45	145.71	NG		

Table 3.4b In-house blood RIs and descriptive statistics for nesting flatback turtles.

Analytes	SI Units	n	Mean	Median	SD	Min	Max	Lower limit (90% CI)	Upper limit (90% CI)	Distrib-ution[b]	Method[c]	Number outliers[d]
PCV	L/L	94	35.7	36	6.1	25	49	26.0 (25.0 - 27.0)	48.3 (46.3 - 49.0)	NG	NP	0
Total solids	g/L	121	53.5	51	10.7	31	80	34.1 (31.0 - 39.0)	77.9 (73.0 - 80.0)	NG	NP	0
Sodium	mmol/L	63	146.7	147	2.8	137	151	138.2 (137.0 - 141.2)	151.0 (150.0 - 151.0)	NG	NP	0
Potassium	mmol/L	63	4.51	4.5	0.42	3.6	5.7	3.67 (3.53 - 3.81)	5.35 (5.20 - 5.49)	G	P	0/1 (D)
Chloride	mmol/L	63	113.0	114	5.1	98	122	102.7 (101.0 - 104.4)	123.4 (121.5 - 125.1)	G	P	0
Glucose	mmol/L	63	3.65	3.9	0.95	1.7	5.7	1.76 (1.70 - 2.20)	5.52 (4.90 - 5.70)	NG	NP	0
BUN	mmol/L	63	1.35	0.5	1.72	0.5	8.8	0.50 (0.50 - 0.50)	8.08 (4.21 - 8.80)	NG	NP	0
HCT	L/L	63	26.5	26.0	4.7	18	42	17.1 (15.5 - 18.7)	35.9 (34.3 - 37.6)	G	P	0/1 (D)

[a] Mean for robust data is the untransformed or transformed mean for the respective data.

[b] Distribution type: NG, non-Gaussian; G, Gaussian; S, symmetrical. Note distribution in parentheses after transformation.

[c] RI calculation method: P, parametric; NP, non-parametric; R, robust; T, transformed; RT, robust transformed. Note descriptive statistics for RIs calculated using transformation are back-transformed values.

[d] Number of extreme/suspect Tukey outliers; (D), Dixon outlier included.

[e] 10-90% percentiles presented due to GLDH low sample size.

* NP used for Gaussian data as other methods did not follow CLSI guidelines or could not be calculated.

Other abbreviations: SI Units, Standard International Units; n, sample size; SD, standard deviation; CI, Confidence Interval; na, not applicable.

Table 3.5a Descriptive statistics for mature foraging flatback turtle laboratory haematological and biochemical RVs.

Analytes	SI Units	n	Mean	Median	SD	Min	Max	Distrib-ution[a]	10 - 90% percentile[b]
PCV	L/L	31	37.8	37	2.99	30	43	NG	35 - 42
Hb	g/L	31	101.0	103	7.28	79	114	NG	92 - 108
RBC	10^{12}/L	31	0.41	0.41	0.03	0.34	0.47	G	
MCHC	g/L	31	267.8	269	13.05	242	294	G	
MCH	pg	31	250.3	252	22.61	206	292	G	
MCV	fL	31	927.2	923	65.92	833	1079	G	
WBC	10^9/L	34	8.68	8.80	2.52	3.30	12.50	G	
Heterophils	%	34	31.4	28.0	12.2	14	71	NG	22 - 46
Heterophils	10^9/L	34	2.57	2.47	0.88	1.22	4.97	G	
Lymphocytes	%	34	47.4	51.0	15.7	8	68	NG	25 - 63
Lymphocytes	10^9/L	34	4.34	4.65	2.12	0.63	7.83	G	
Monocytes	%	34	11.0	10.0	5.5	2	23	G	
Monocytes	10^9/L	34	0.92	0.74	0.58	0.21	2.82	NG	0.39 - 1.62
Eosinophils	%	34	9.9	9.0	6.5	1	28	G	
Eosinophils	10^9/L	34	0.81	0.66	0.55	0.12	2.30	NG	0.26 - 1.54
Basophils	%	32	0.3	0.0	0.6	0	2	NG	0 - 1
Basophils	10^9/L	32	0.03	0.00	0.06	0.00	0.23	NG	0 - 0.11
H: L ratio		34	0.92	0.52	1.27	0.24	6.75	NG	0.38 - 1.12
CK	U/L	32	1204.3	1140	706.1	198	3135	G	
AST	U/L	32	232.5	206	103.7	114	594	NG	150 - 346
ALT	U/L	32	7.9	7	3.5	3	20	NG	5 - 12
ALP	U/L	31	79.2	77	28.7	35	174	NG	53 - 116
Bilirubin T	µmol/L	32	2.13	2.0	0.51	1	3	NG	1.64 - 2.89
BUN	mmol/L	32	19.23	19.2	3.79	12	31	G	
Bile acids	µmol/L	32	1.8	2	1.0	0	5	NG	1 - 3
Uric acid	mmol/L	32	0.077	0.077	0.019	0.033	0.115	G	
Glucose Fl-Ox	mmol/L	31	2.70	2.7	0.54	1.4	3.9	G	
Cholesterol	mmol/L	32	5.45	4.4	2.92	2.3	15.0	NG	3.0 - 8.6
Triglyceride	mmol/L	32	6.82	2.8	7.90	0.8	30.9	NG	1.1 - 18.7
Sodium	mmol/L	32	151.6	151	4.0	144	160	G	
Potassium	mmol/L	32	3.81	3.9	0.42	2.4	4.5	NG	3.4 - 4.3
Chloride	mmol/L	32	120.5	120	5.6	108	130	G	
Total protein	g/L	32	44.8	44	7.2	32	68	G	
Albumin	g/L	32	13.5	13	2.4	8	20	G	
Globulin	g/L	32	31.3	31	5.1	22	48	G	
Calcium	mmol/L	32	2.68	2.01	1.34	1.58	6.43	NG	1.61 - 4.29
Phosphorus	mmol/L	32	2.08	1.82	0.78	0.97	3.76	NG	1.33 - 3.52
Ca: P ratio		32	1.28	1.16	0.34	0.81	2.33	NG	0.95 - 1.65
Magnesium	mmol/L	32	3.41	3.14	0.67	2.55	4.97	NG	2.75 - 4.39
Iron	µmol/L	32	6.80	5.5	4.02	1.5	15.8	NG	3.0 - 13.1
LDH	U/L	32	671.0	611	185.6	414	1009	NG	469 - 926
GLDH	U/L	32	32.60	22.8	30.30	6.9	156.2	NG	10.8 - 59.7

Table 3.5b Descriptive statistics for mature foraging flatback turtle in-house blood results.

Analytes	SI Units	n	Mean	Median	SD	Min	Max	Distrib-ution[a]	10 - 90% percentile[b]
PCV	L/L	36	38.7	38	2.7	34	45	G	
Total solids	g/L	35	53.5	49	15.6	31	102	NG	39 - 75.6
Sodium	mmol/L	6	146.5	146	4.0	143	154	G	
Potassium	mmol/L	6	4.02	4.1	0.29	3.6	4.4	G	
Chloride	mmol/L	6	118.5	118	2.7	117	124	NG	117 - 121
Glucose	mmol/L	6	2.40	2.5	0.15	2.1	2.5	NG	2.25 - 2.5
BUN	mmol/L	6	19.65	19.1	2.73	16.6	23.8	G	
HCT	L/L	6	28.5	29	2.5	25	31	G	

[a] Distribution type: NG, non-Gaussian; G, Gaussian.

[b] 10-90% percentiles calculated for non-Gaussian data.

3.3.3 Effect of life stage

The final dataset (i.e., after outlier removal) for nesting and foraging populations had significantly different values for 36/44 (81.8%) of blood parameters (Table 3.6). Nesting turtles had statistically higher Hb, MCHC, MCH, MCV, absolute and differential heterophil counts, differential eosinophil count, H: L ratio, bilirubin T, glucose (Fl-Ox), cholesterol, triglyceride, sodium, potassium, calcium, phosphorus, and magnesium. While foraging turtles had significantly higher PCV, RBC, absolute and differential lymphocyte counts, absolute and differential monocyte counts, absolute and differential basophil counts, CK, AST, ALT, ALP, BUN, uric acid, bile acid, chloride, Ca: P ratio, LDH, and in-house PCV. Mean +/- SD or median values plus 10-90% percentiles are presented to facilitate comparison noting these are all untransformed values. The i-STAT results could not be evaluated due to insufficient foraging sample size following removal of outliers.

Further analysis of life stage (nesting vs foraging) for females found only 28/44 (63.6%) were significantly different, as opposed to 36/44 (81.8%) for nesting vs foraging turtles regardless of sex. In the female only analysis, only TWCC became significant (higher in foraging female turtles), while Hb, AST, ALT, bilirubin T, uric acid, cholesterol, triglyceride, calcium, and LDH became non-significant.

Table 3.6 Blood parameters for flatback turtles based on life stage (final nesting or foraging RVs) with mean +/- SD and t-test results for parametric data and median (10-90% percentiles) and Mann-Whitney U test results for non-parametric data.

Analyte[1]	Foraging Mean +/- SD or Median (10-90% percentiles)	Nesting Mean +/- SD or Median (10-90% percentiles)	p	df	t	W
PCV	37 (35 - 42)	34 (30 - 42)	0.006			1320
Hb	103 (92 - 108)	109 (95 - 128)	0.01			655.5
RBC	0.41 (0.37 - 0.45)	0.20 (0.17 - 0.40)	<0.001			1785.5
MCHC	269 (251 - 283)	323 (292 - 339)	<0.001			136
MCH	252 (218 - 277)	462 (314 - 619)	<0.001			50
MCV	923 (844 - 1005)	1450 (950 - 2235)	<0.001			184
WBC	8.68 +/- 2.52	7.69 +/- 2.83	**0.081**	112	1.76	
Heterophils						
Differential	28.0 (22.0 - 45.5)	55.0 (32.3 - 79.0)	<0.001			327
Absolute	2.47 (1.62 - 3.69)	4.40 (2.01 - 7.07)	<0.001			625
Lymphocytes						
Differential	47.4 +/- 15.7	26.8 +/- 14.6	<0.001	116.00	6.79	
Absolute	4.65 (1.15 - 6.66)	2.18 (0.33 - 4.01)	<0.001			2183
Monocytes						
Differential	10.0 (4.3 - 17.0)	4.0 (1.0 - 9.7)	<0.001			2322.5
Absolute	0.74 (0.39 - 1.62)	0.32 (0.06 - 0.76)	<0.001			2203.5
Eosinophils						
Differential	9.91 +/- 6.46	12.98 +/- 6.4	0.022	60	-2.34	
Absolute	0.66 (0.26 - 1.54)	0.92 (0.25 - 1.80)	**0.136**			1119
Basophils						
Differential	0.0 (0.0 - 1.0)	0.0 (0.0 - 0.0)	<0.001			1599
Absolute	0.00 (0 - 0.11)	0 (0 - 0)	<0.001			1521
H: L ratio	0.52 (0.38 - 1.12)	1.68 (0.73 - 4.29)	<0.001			318
CK	1140 (414 - 2115)	457 (220 - 1871)	<0.001			1925
AST	206 (150 - 346)	180 (111 - 269)	0.017			1752.5
ALT	7 (5 - 12)	6 (3 - 9)	0.004			1785
ALP	77 (53 - 116)	53 (38 - 73.6)	<0.001			2058.5
Bilirubin T	2.0 (1.6 - 2.9)	2.4 (1.1 - 5.9)	0.033			1025
BUN	19.2 (14.9 - 22.6)	1.8 (0.8 - 3.7)	<0.001			2752
Bile acids	2 (1 - 3)	1 (1 - 2)	0.044			1636
Uric acid	0.077 (0.054 - 0.103)	0.063 (0.044 - 0.082)	<0.001			1958.5
Glucose Fl-Ox	2.7 (2.0 - 3.4)	3.8 (2.51 - 4.8)	<0.001			209.5
Cholesterol	4.4 (3.0 - 8.6)	7.0 (4.5 - 10.7)	<0.001			772.5
Triglyceride	2.8 (1.1 - 18.7)	7.1 (2.7 - 13.2)	0.006			917.5
Sodium	151 (148 - 157)	153 (150 - 157)	0.038			1035
Potassium	3.81 +/-0.42	4.68 +/- 0.45	<0.001	117	-9.34	
Chloride	120 (114 - 126)	111 (105.5 - 119)	<0.001			2394.5
Total Protein	44.8 +/- 7.2	45.6 +/- 6.8	**0.508**	117	-0.66	
Albumin	13 (11 - 16)	14 (10.5 - 18)	**0.388**			1234
Globulin	31.3 +/- 5.1	31.7 +/- 4.7	**0.626**	117	-0.49	
Calcium	2.68 +/- 1.34	3.87 +/- 1.03	<0.001	117	-5.17	
Phosphorus	1.82 (1.33 - 3.52)	3.52 (2.44 - 4.63)	<0.001			321.5
Ca: P ratio	1.16 (0.95 - 1.65)	1.08 (0.87 - 1.39)	0.017			1771.5
Magnesium	3.14 (2.75 - 4.39)	4.24 (3.32 - 4.94)	<0.001			544
Iron	5.5 (3.0 - 13.1)	8.0 (4.0 - 11.1)	**0.080**			1087.5
LDH	611 (469 - 926)	527 (350 - 954)	0.006			1832
GLDH	22.8 (10.8 - 59.7)	22 (7.0 - 76.1)	**0.507**			417.5
In-house PCV	38 (36 - 43)	36 (28 - 45)	0.001			2304
Total solids	49 (39 - 76)	51 (40 - 68)	**0.366**			1904.5

[1] No availability of glucose Li-Hep due to missing values from one group, or i-STAT comparisons due to foraging i-STAT sample size <7.

3.3.4 Effect of sex

Difference between sexes was apparent with foraging females having significantly higher Hb (W=172.5, p=0.035), TWCCs (t(32)=2.58, p=0.015), absolute and differential lymphocyte counts (t(32)=3.4, p=0.002 and W=228.5, p=0.003 respectively), cholesterol (W=199.5, p=0.006), triglyceride (W=205, p=0.003), albumin (t(30)=2.49, p=0.019), calcium (W=195.5, p=0.009), phosphorus (W=182, p=0.034), iron (W=209.5, p=0.002), and in-house TPS (W=222, p=0.017) than males. Males had higher values for differential heterophil counts (W=70.5, p=0.013), differential eosinophil counts (t(32)= -2.16, p=0.039), higher H: L ratio (W=59, p=0.006), CK (t(30)= -2.28, p=0.03), AST (W=59, p=0.012), uric acid (t(30)= -2.53, p=0.017), sodium (t(30)= -3.16, p=0.004), chloride (t(30)= -2.45, p=0.02), and LDH (W=51.5, p=0.005) (see Appendix 14 for details).

3.3.5 Effect of rookery and season

We found significant differences in 25/48 (52.1%) analytes between Thevenard Island and Eighty Mile Beach rookery. Nesting turtles at Eighty Mile Beach had statistically higher differential eosinophil count (t(82)=2.59, p=0.011), ALT (W=1227, p<0.001), BUN (W=1491, p<0.001), glucose (Li-Hep) (t(46)=2.92, p=0.005), potassium (t(84)=4.95, p<0.001), chloride (W=1693.5, p<0.001), phosphorus (t(84)=2.42, p=0.018), and magnesium (t(83)=2.34, p=0.022), as well as i-STAT sodium (W=651.5, p=0.009), potassium (t(61)=5.09, p<0.001), chloride (t(61)=9.03, p<0.001), and BUN (W=686, p<0.001). Thevenard Island nesters had higher Hb (W=343.5, p=0.038), differential and absolute heterophil counts (t(77)= -2.23, p=0.029 and W=582.5, p=0.04, respectively), bilirubin T (W=351, p<0.001), uric acid (W=660.5, p=0.023), triglyceride (t(75)= -2.94, p=0.004), total protein (t(84)= -4.07, p<0.001), albumin (W=536, p<0.001), globulin (t(84)= -4.04, p<0.001), Ca: P ratio (W=529.5, p<0.001), iron (W=237, p<0.001), TPS (W=1032, p<0.001), and i-STAT HCT (t(36)= -3.38, p=0.002) (see Appendix 15 for details).

For nesting turtles, analysis based on trip (n=6) and season (n=2) subset by location (n=2 for each rookery) revealed a significant number of differences by trip, and within rookery differences for different seasons. As mentioned 25/48 (52.1%) analytes were statistically different between Thevenard Island and Eighty Mile Beach rookery. I examined season by

rookery by combining all trips from each rookery for both the 2016/2017 and 2017/2018 seasons and found that 25/46 (54.3%) analytes were statistically different for Eighty Mile Beach and 10/40 (25.0%) were statistically different for Thevenard Island. Statistically significant analytes between and within (both) rookeries included potassium, albumin, phosphorus, while MCHC, MCV, and H: L ratio differed within rookeries but were the same between rookeries. Further seasonally different analytes for Eighty Mile Beach include laboratory PCV, heterophil differential and absolute counts, lymphocyte differential count, uric acid, cholesterol, triglyceride, total protein, globulin, sodium, calcium, magnesium, iron, LDH, field PCV, TPS, as well as i-STAT potassium, chloride, and glucose. While other statistically different analytes for Thevenard Island include monocyte differential count, eosinophil absolute count, chloride, and Ca: P ratio.

For foraging turtles, statistical testing could only be performed for Roebuck Bay field trip one and two (same season 2018 but two months apart). Only 9/44 (20.5%) analytes were different including MCHC, MCH, MCV, eosinophil differential and absolute counts, ALT, BUN, and magnesium.

3.3.6 Temporal effects

When comparing time to analysis (<48 hours and <60 hours), centrifugation (<6 hours and <8 hours), and i-STAT analysis (<6 hours and <8 hours), statistical testing was only possible for nesting animals as foraging animal sample sizes were too small. Consequently, there were no significant differences except for differential lymphocytes which decreased with time (n=14, p=0.024), and AST which paradoxically decreased with time (n=11, p=0.009), as well as elevated glucose with increased time for i-STAT analysis (n=6 only, p=0.037), while phosphorus and calcium were paradoxically decreased with increased time (n=10, p=0.006 and p=0.018 respectively). Furthermore, correlation with time was poor for all analytes (rho <0·59).

There were significant increases in H: L ratio for nesting turtles; on the beach for >60 minutes (n=83, W=370, p=0.032), >1 minute restraint prior to blood collection (n=78, W=512, p=0.028), time between capture and blood collection >5 minutes (n=79, W=158, p=0.016), and ≥3 attempts for blood collection (n=84, W=30, p=0.002). However, for foraging turtles,

although not significant, there were increases in H: L ratio for holding ≥10 minutes (n=34, W=69, p=0.055), and >1 minute restraint prior to blood collection (n=34, W=42, p=0.06).

3.3.7 Body condition

Only 1/195 (0.5%) turtles was not considered to be in good body condition (less than 4/5 body condition score), as well as one duplicate foraging turtle excluded from the analysis. During the field examination, 5/146 (3.4%) nesting and 5/49 (10.2%) foraging turtles were considered to have abnormal health (plus the duplicate foraging turtle) and were subsequently excluded from RI development. Four of these individuals (plus the duplicate) were visually identified as having diminished health during physical examination (e.g., body condition score less than 4/5, heavy barnacle load, active infections), while the remaining six were considered to have questionable health post-analysis of blood results. No cases of fibropapillomatosis were detected.

3.3.8 Intraerythrocytic haemoparasite and other haematologic abnormalities

During blood film examination, intraerythrocytic vacuoles were detected and on closer inspection, one foraging flatback turtle had mature haemoparasitic life stages within RBCs (Figure 3.3a and 3.3b, and Figure 3.4), preliminarily identified as a new species of *Haemocystidium*. Because of the low haemoparasite detection rate, these intraerythrocytic vacuoles were instead analysed as a potential proxy for haemoparasites due to their frequent association with haemoparasites (Trocini 2013, Austen et al. 2020). When comparing the different life stages (of the whole dataset, excluding duplicates), these vacuoles were more prevalent in foraging turtles (44/49, 89.8%) compared to nesting turtles (26/146, 17.8%; OR=40.62, 95% CI: 15.9-126.4, p <0.001). The five foraging turtles with no vacuoles were all at Eighty Mile Beach (5/7, 71.4%), with 100% Roebuck Bay flatback turtles having vacuoles. There was no difference when comparing the impact of sex on presence of vacuoles in the foraging turtles (males 17/19, 89.5%; females 25/28, 89.3%).

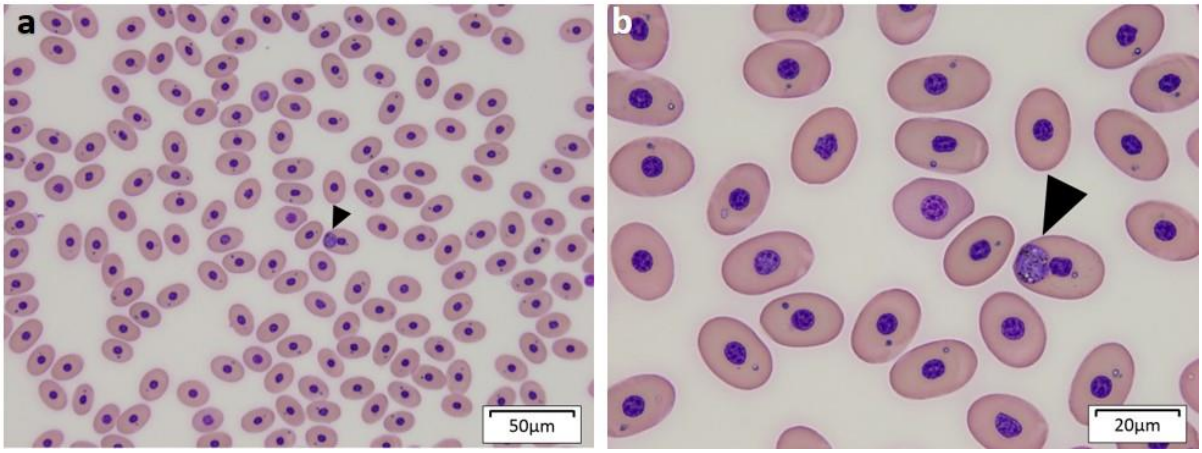


Figure 3.3 (a, b) Flatback turtle blood film (x400, x1000) showing macrogamont life stage (arrowhead) of *Haemocystidium* parasite.

Grading of the vacuoles found 41/49 (83.7%) of foraging turtles had high numbers of vacuoles, while 3/49 (6.1%) had low numbers, whereas 1/146 (0.7%) nesting turtles had a heavy burden and 23/146 (15.8%) had a low burden. When examining presence of basophils (n=23) and vacuoles (foraging and nesting animals combined of the whole dataset), the odds of vacuoles were significantly higher in turtles with basophils (OR=8.40, 95% CI: 3.2-26.60, p<0.001).

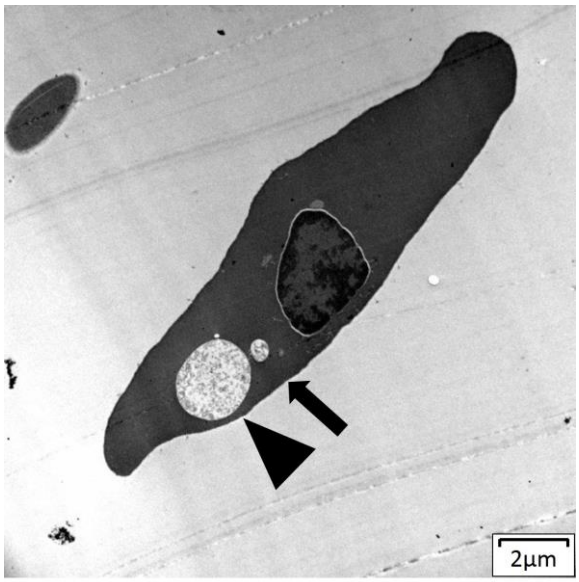


Figure 3.4 Electron microscopy flatback turtle RBC with intracellular microgamont *Haemocystidium* parasite (arrowhead) and intracytoplasmic vacuole (or immature ring stage) (arrow).

When examining health status (abnormal n=10, normal n=185) through physical examination or extreme outlier blood results, and presence of vacuoles (foraging and nesting animals combined), there was no significant relationship between health status and presence of vacuoles (OR=2.84, 95% CI: 0.8-10.4, p=0.116).

Only foraging turtles had any type of blood film abnormalities (14/49, 28.6%), such as polychromasia and/or anisocytosis, with half mild and half moderate grading of changes (7/14, 50.0% for each). Sample sizes were too small for any comparisons with health status or other health-related variables.

3.3.9 Comparison of laboratory and field techniques

Using the combined dataset (nesting and foraging), Bland-Altman plots were developed to test the congruence of field and laboratory techniques. Only two analyte results were parametric and therefore met the Bland-Altman assumptions (Figure 3.5). Laboratory sodium and i-STAT sodium were significantly different and the Bland-Altman plot showed a constant positive bias of 5.49 mmol/L where i-STAT values were lower than laboratory values (3.92% outside limits of agreement), and there was also a small positive proportional bias with increasing bias at higher average plasma concentrations (as values became further from zero). Measurement methods for Li-Hep and Fl-Ox glucose compared to i-STAT glucose were both significantly different and had a small positive proportional bias, with Li-Hep showing a slightly lower constant bias than Fl-Ox (0.41 mmol/L and 0.48 mmol/L respectively), and no values outside limits of agreement (see Appendix 16 for Fl-Ox Bland-Altman plot). For both Bland-Altman plots, <5% fell outside limits of agreement. Bland-Altman graphs for the method comparisions for the remaining analytes with non-parametric differences are located in Appendix 16 as the assumption of parametric distribution was not met.

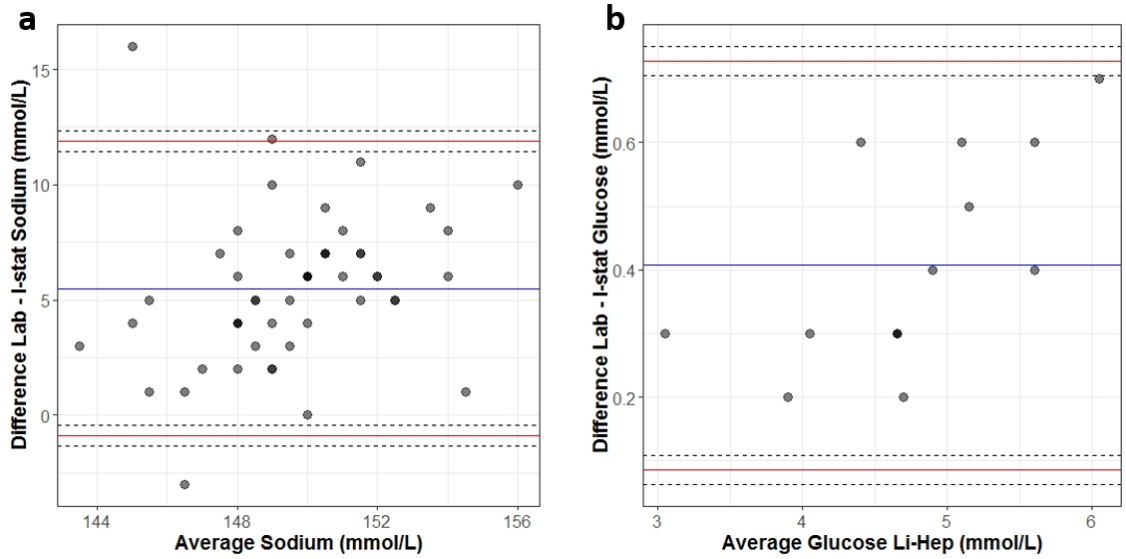


Figure 3.5 (a, b) Bland-Altman plots for nesting turtles with parametric difference data for sodium (n=51) and glucose Li-Hep (n=14) showing mean difference or bias (blue line) between the two methods and limits of agreement (red lines) set as mean difference +/- 1.96sd, with 95% CIs for these limits (dotted lines).

For all other analytes (with non-parametric distribution), Passing-Bablok linear regression plots were used to test congruence (Figure 3.6). The regressions showed statistically significant constant and/or proportional bias for sodium, chloride, total protein/TPS, and PCV/i-STAT HCT. Laboratory sodium was constantly and proportionally higher than i-STAT sodium. The y-intercept was 57.57 mmol/L indicating constant bias (95% CI, 12.39-86.0), and the slope of the regression was 0.59 (95% CI, 0.4-0.88) and consistent with proportional bias found with the Bland-Altman plot. When considering total protein/TPS, TPS was both constantly and proportionally higher than laboratory total protein. The y-intercept of the regression equation indicating constant bias was -20.25 g/L (95% CI, -28.2 to -14.75). The slope of the Passing-Bablok was 1.63 (95% CI, 1.5-1.8), indicating significant proportional bias. Moreover, there was increasing disparity at higher concentrations. Laboratory chloride and PCV were also constantly higher than their respective i-STAT values, with a y-intercept of -23.8 mmol/L (CI 95%, -51.15 to -1.0) and -8 L/L (95% CI, -24 to -2.31), respectively. Passing-Bablok plots for BUN, potassium, and Li-Hep glucose all had excellent correlation (r=1.0), PCV (laboratory and field), total protein and TPS, chloride as well as Fl-Ox glucose had good correlation (r=0.9, r=0.89, r=0.86 and r=0.81, correspondingly), while i-STAT HCT correlations (lab and field PCV) were fair (r=0.62 and r=0.78, respectively), and sodium was the only poor correlation (r=0.44) (Bauer and Moritz 2008). Field and laboratory PCV

correlation improved from fair (r=0.67) to good (r=0.9) during data clean-up and the removal of PCV values with a difference >20%. While correlations were mostly good, when examining these plots in conjunction with Bland-Altman (see Appendix 16 for additional plots), the results are clearly not equivalent to the laboratory results.

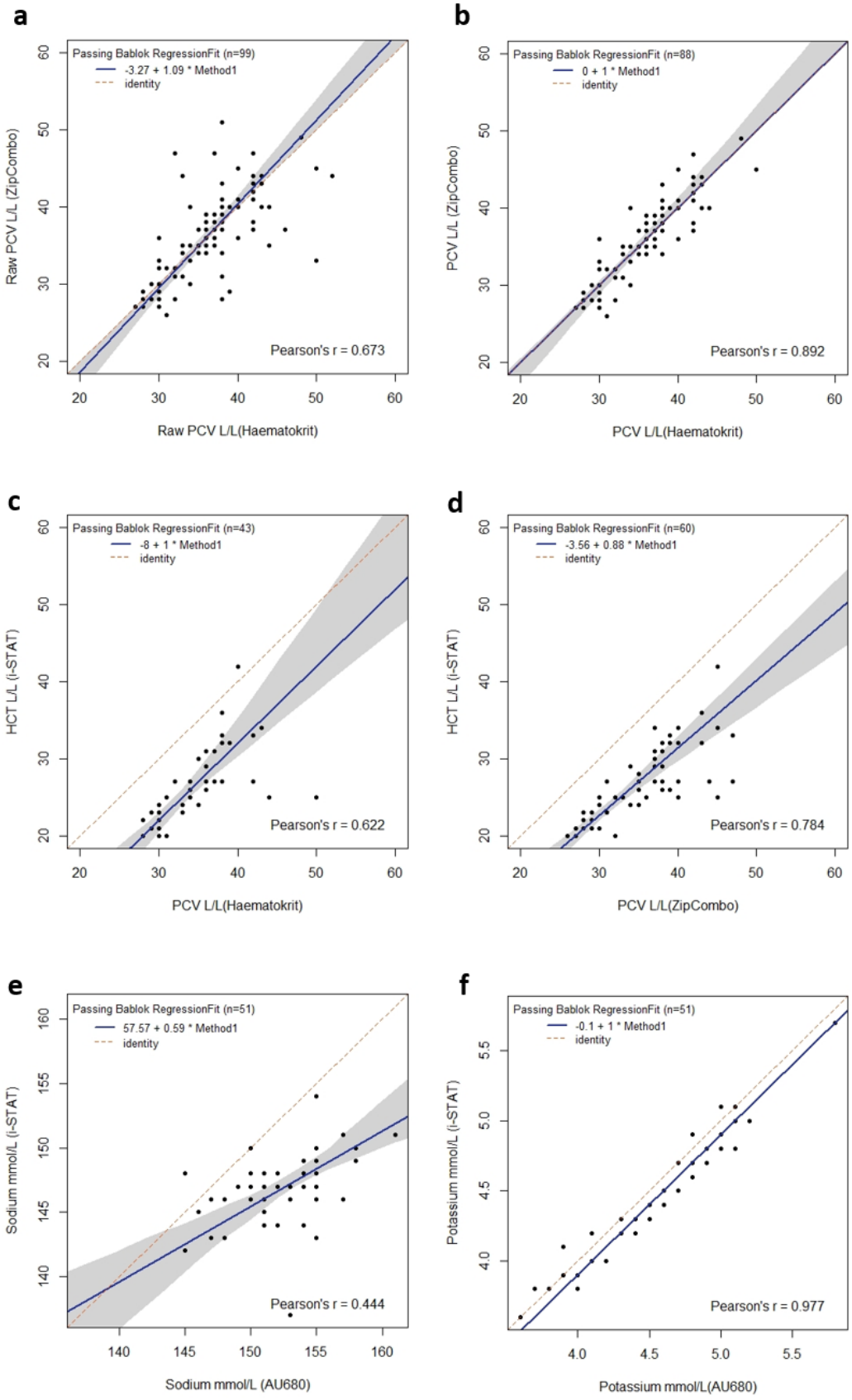
3.4 Discussion

3.4.1 Reference intervals and outliers

This study reports the first RIs for flatback turtles, specifically nesting turtles, and the first blood RVs for foraging flatback turtles. While other flatback turtle blood values have been reported (Sperling et al. 2007, Guinea 2016, Scheelings et al. 2020), these are the first to follow IFCC-ASVCP guidelines in establishing RIs, including having an adequate sample size, utilising important outlier detection techniques, setting exclusion criteria (e.g., haemolysis) and implementing appropriate statistical analyses (Friedrichs et al. 2012).

One hundred and twenty is the minimum sample size recommended for partitioning, except for certain selection criteria likely to cause significant differences for example age, and sex. The decision to present separate values for nesting and foraging turtles was supported by statistical analysis and the obvious physiological differences for the nesting turtle, akin to pregnancy, an exclusion criterion frequently applied for partitioning (Friedrichs et al. 2012). Conversely, statistical significance does not always implicate clinical significance or that partitioning should be performed (Atkins et al. 2010).

While our exclusion criteria typically followed ASVCP guidelines and was supported by previous research, field conditions precluded the generally accepted timeframes of 48 hours to laboratory analysis and six hours to centrifugation. Statistical analyses revealed the only clinically important difference for extending these timeframes, specifically, 60 hours to haematological analysis was a statistically significant decrease in lymphocyte differential count (28.0% vs 18.2%). Sample degradation is known to affect cell morphology and differential cell counts (Fudge 2000b), but since only one analyte was affected, it was decided to include these extended timeframes as no differences were detected for absolute lymphocyte count.


a
Passing Bablok RegressionFit (n=99)
-3.27 + 1.09 * Method1
identity
Pearson's r = 0.673
Raw PCV L/L (ZipCombo)
Raw PCV L/L(Haematokrit)
b
Passing Bablok RegressionFit (n=88)
0 + 1 * Method1
identity
Pearson's r = 0.892
PCV L/L (ZipCombo)
PCV L/L(Haematokrit)
c
Passing Bablok RegressionFit (n=43)
-8 + 1 * Method1
identity
Pearson's r = 0.622
HCT L/L (i-STAT)
PCV L/L(Haematokrit)
d
Passing Bablok RegressionFit (n=60)
-3.56 + 0.88 * Method1
identity
Pearson's r = 0.784
HCT L/L (i-STAT)
PCV L/L(ZipCombo)
e
Passing Bablok RegressionFit (n=51)
57.57 + 0.59 * Method1
identity
Pearson's r = 0.444
Sodium mmol/L (i-STAT)
Sodium mmol/L (AU680)
f
Passing Bablok RegressionFit (n=51)
-0.1 + 1 * Method1
identity
Pearson's r = 0.977
Potassium mmol/L (i-STAT)
Potassium mmol/L(AU680)

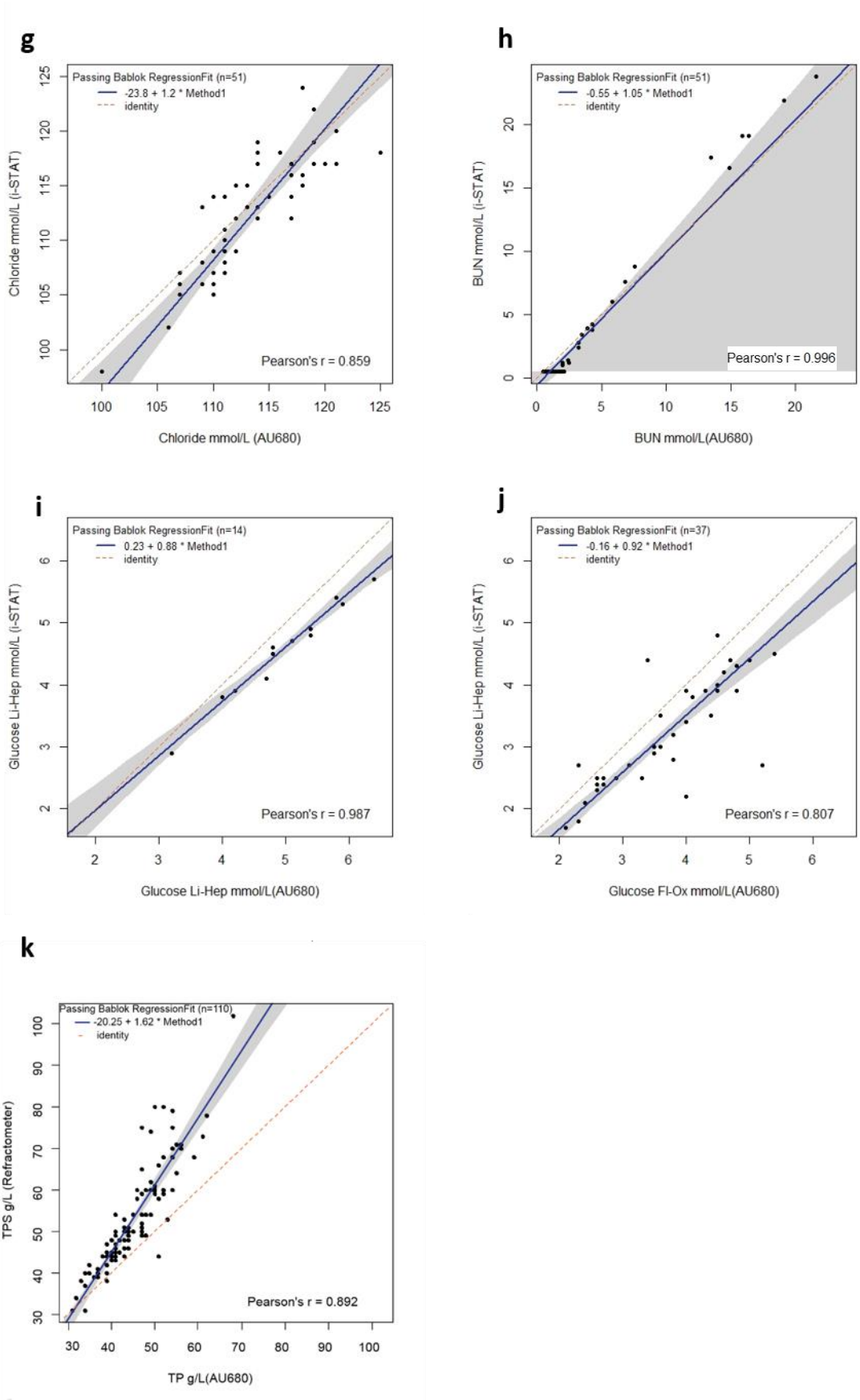


Figure 3.6 (a–k) Passing-Bablok method comparison plots for PCV (lab vs field vs i-STAT) as well as laboratory vs i-STAT BUN, sodium, potassium, chloride and glucose (Li-Hep and Fl-Ox) and total

protein/TPS showing the regression line (blue line), 95% CI for the regression line (grey shaded area), the identity or equality line (red dashed line), as well as the regression equation and Pearson's correlation co-efficient Note BUN Passing-Bablok plot CI affected by conversion of i-STAT BUN values of "<1" to "0.5".

While we have used outlier detection methods, and followed ASVCP guidelines, detection is not guaranteed, for example, multiple outliers at each extremity can mask outliers (Friedrichs et al. 2012). The best option is to critically examine each suspect value for any reason for exclusion. While emphasis is always on retention, removal of outliers rests with the clinician's best judgment, comparison with other established RIs, and ultimately consideration of the uncertainty surrounding the history of wild sea turtle health (Geffré et al. 2009, Friedrichs et al. 2012). If there was no justification for removal, outliers were not removed, for example, as basophils were rarely detected and >0 was regarded as an outlier, due to their rarity, there is a possibility these cells were missed on routine estimated TWCCs.

As reported in the literature, we found significant differences between blood values for different boundary conditions including life stage (e.g., foraging or nesting), rookery, sex, and season (Bolten and Bjorndal 1992, Hasbún et al. 1998, Samour et al. 1998, Stamper et al. 2005, Hamann et al. 2006, Santoro and Meneses 2007, Whiting et al. 2007a, Casal et al. 2009, Deem et al. 2009, Harris et al. 2011, Perrault et al. 2012, Kelly et al. 2015, Sözbilen and Kaska 2018). Unfortunately, statistical testing was not available for some boundary groups including maturity, restraint, satellite tracked animals from similar home-ranges, or a number of health parameters (e.g., significant carapace or flipper deficits, body condition or questionable health status detected on physical examination or through clinical pathology results), due to inadequate sample size.

3.4.2 Life stage

Similar to other studies, nesting turtles had significantly higher cholesterol, triglyceride, calcium, phosphorus, magnesium, glucose, and haemoglobin, likely related to vitellogenesis and folliculogenesis (and other physiological changes related to breeding) compared to foraging turtles (Dessauer 1970, Hasbún et al. 1998, Hamann et al. 2002, Casal et al. 2009, Deem et al. 2009, Perrault and Stacy 2018, Sözbilen and Kaska 2018).

Adult foraging flatback turtles had higher PCV and RBC compared to nesting turtles, similar to differences found in other species (Innis et al. 2010, Harris et al. 2011). Even though erythropoiesis decreases in winter when most foraging turtles were sampled, these values are proposed to be higher in foraging animals in good nutritional status due to feeding and lower in nesting turtles due to physiological effects on bone marrow and stress of nesting (Harris et al. 2011, Stacy et al. 2011, Kelly et al. 2015, Perrault et al. 2016, Perrault and Stacy 2018).

As found in some other studies, heterophils were higher in nesting turtles (Casal et al. 2009, Deem et al. 2009, Sinaei et al. 2019), while lymphocytes were the predominant WBC in foraging turtles (Day et al. 2007, Flint et al. 2010c, Lewbart et al. 2014). Other studies found the reverse (Samour et al. 1998, Harris et al. 2011), suggesting the higher heterophils in foraging leatherback turtles could be stress related to capture. Reptile lymphocyte production has been reported to seasonally decrease in winter, however, this was not the case in our study (Zapata et al. 1992). Following on from this, H: L ratios were higher in nesting turtles compared to foraging turtles.

Blood urea nitrogen and uric acid were significantly higher in foraging turtles compared to nesting turtles, most likely related to the fasting state during nesting season (Casal et al. 2009, Deem et al. 2009, Perrault et al. 2012). Almost all enzymes were higher in foraging turtles collected over the autumn-winter seasons compared to nesting turtles sampled in summer. On the contrary, Christopher et al. (1999) reported a decrease in numerous enzymes during winter in other reptile species which hibernate. While CK and AST are primarily found in muscle, most other enzymes (e.g., ALP) have a wide tissue distribution (Anderson et al. 2013, Petrosky et al. 2015) and higher levels in foraging animals, as found in our study, have previously been reported to be a result of increased activity (when foraging), but could also possibly be related to stress surrounding capture and restraint. Alternatively, it could be an artefact and associated with prolonged phlebotomy (Anderson et al. 2011), elevated storage temperature (possible under field conditions) or delayed separation (Thoresen et al. 1992, Eisenhawer et al. 2008, Eshar et al. 2018), and even if not grossly observable, haemolysis (Christopher et al. 1999, Harris et al. 2011). Generally, AST

and LDH enzymes are reported to increase with haemolysis, while changes in CK, ALT, and ALP are variable (Benson et al. 1999, Murray 2000, Hawkins et al. 2006).

Electrolytes significantly higher in nesting turtles included sodium by a difference of 1.4 mmol/L (0.9%) and potassium by 0.87 mmol/L (20.5%), while chloride was statistically lower by an average difference of 8.7 mmol/L (7.5%) when compared to the foraging group. Increases in these electrolytes in foraging animals are reportedly due to feeding (Stamper et al. 2005) and simultaneous ingestion of seawater (Lutz 1997), which supports our chloride but not the sodium results.

Protein is a useful analyte, and frequently used to assess health and nutritional status, and in disease studies. For example a negative correlation of fibropapillomatosis to protein and protein fractions has been reported (Aguirre et al. 1995, Work et al. 2001). Although laboratory machines are frequently used to analyse albumin and globulin, our results were presented to compare with other studies, despite the fact that electrophoresis is the preferred method of analysis.

The lack of statistically significant differences between protein in the various life stages (i.e., nesting or foraging) differed to some studies where all protein fractions were higher in nesting turtles (Casal et al. 2009, Deem et al. 2009), and to others where all higher values were detected in foraging animals (Harris et al. 2011). While it is reported that various sea turtle species, including leatherback turtles, tend to have higher protein at the start of the nesting season compared to the end (Goldberg et al. 2013, Perrault et al. 2014), protein can also increase with feeding. Anecdotally blooms of tomato jellyfish were observed in Roebuck Bay and many turtles were seen consuming jellyfish, albeit low as a source of protein (Machovsky-Capuska and Raubenheimer 2020).

3.4.3 Sex

With regards to differences between the sexes, male turtles had statistically higher AST, uric acid, sodium, and CK, which has been previously reported (Bolten and Bjorndal 1992, Hasbún et al. 1998, Innis et al. 2010), as well as LDH, chloride, and H: L ratio. Higher uric acid in foraging males compared to foraging females could potentially be related to higher uric acid clearance in females observed in other species (Lamb and Jones 2018). This however

was in direct contrast to Hamann et al. (2006) who found higher uric acid in females (n=4 only). These higher enzyme levels reported could relate to increased muscle catabolism possibly associated with capture and restraint or male-specific behaviour, such as intra-specific aggression. As previously mentioned, although it was not breeding season, many of the foraging animals migrated and were possibly of pre-breeding status at the time of sampling, but small sample sizes precluded any statistical testing.

Many of the analytes higher in nesting females (e.g., cholesterol, triglyceride, calcium, phosphorus, and Hb) were also higher in foraging females when compared to males. Pre-vitellogenic females likely contributed to these differences with breeding migrations (of both males and females) identified through satellite tracking, enabling detection of three pre-vitellogenic females (Seaturtle.org 2018a). Flatback turtles are known to migrate for breeding as frequently as every year (Limpus 2007). Similar to the assumption nesting turtles are healthy, it was assumed turtles which migrated for breeding were also in good health, highlighting additional unknowns with regards to blood values in sea turtles.

3.4.4 Rookery and seasonal differences

Eighty Mile Beach had more variation between seasons when compared to Thevenard Island. The first season at Eighty Mile Beach had statistically higher lipids, proteins, PCV, heterophils (but lower lymphocytes), H: L ratio, uric acid, potassium, calcium, phosphorus, magnesium, iron and not statistically but higher estimated TWCC, AST, and ALT. While the second season had statistically higher LDH, sodium, lymphocytes and higher CK, BUN, ALP and chloride. The reason for these seasonal differences is unknown, however, it could be related to environmental, or seasonal differences such as improved nutrition in the lead up to breeding. The impact of seasonal effects on health has been demonstrated in the marine megafauna at Shark Bay, WA. Here green turtle health parameters including reduced size and lower body condition score continued for two years after a marine heatwave due to its effect on seagrass, the main food source for green sea turtles (Thomson et al. 2015). The higher sodium (and chloride) in the second season could also be related to low rainfall comparative to the first season and the flatback turtles' osmoregulation (Dessauer 1970, Lutz 1997, Bureau of Meteorology 2016, 2017).

Our study showed electrolytes varied across the various boundary conditions, especially potassium, showing additional statistically significant differences across rookeries and within both rookeries over different seasons. This could be related to temperature, as a trend of decreasing potassium at lower temperatures reported in other studies was detected here (Lutz and Dunbarcooper 1987, JPL OurOcean Project 2010).

Protein was found to be significantly different between rookeries, with turtles from Thevenard Island having higher levels of all protein fractions. While these are different populations, coming from different foraging grounds, turtles at Thevenard Island were also larger than Eighty Mile Beach turtles, and protein is reported to correlate positively with size (Hasbún et al. 1998, Whiting et al. 2007a, Delgado et al. 2011).

3.4.5 Size and age-class

While nesting turtle standard carapace measurements have been established (Limpus 2007), typical growth rates and age-classes available for other species do not exist for flatback turtles. With no size data for foraging animals, their expected size at maturity remains unclear, particularly for males. Additionally, the foraging site is further complicated being comprised of mixed genetic stock. Satellite tracking data revealed breeding migrations from Roebuck Bay as far south as Point Sampson (approximately 600 km), and over 800 km north of Cape Londonderry (Seaturtle.org 2018a).

The three immature flatback turtles all had the lowest CCL, CCW, and weight measurements. The next smallest foraging turtles were longer than mature nesting females from Thevenard Island but were initially considered to be sub-adults, with anecdotally lower blood values similar to juveniles or values (such as low iron 1.5 µmol/L) which were initially considered "unhealthy". However, some of these turtles fitted with satellite trackers proceeded to undergo breeding migrations. Hasbún et al. (1998) also found iron was correlated to body size, while Whiting et al. (2007a) reported no correlation.

3.4.6 Regional differences

Some studies show separation of boundary conditions is not required for some RVs for example age and sex, and that these may be valid over other geographic regions (Flint et al.

2010c). Deem et al. (2009) developed separate baseline RIs for foraging and nesting loggerhead turtles, whereas Flint et al. (2010b), suggested established green and loggerhead RIs were generally similar in the same region (regardless of age and sex) and could be comparable in other regions (Flint et al. 2010c). This was in direct contrast to results found by Whiting et al. (2007a) which recommended presentation of separate values in different geographic regions where diet and habitat may differ between foraging areas. However, assessing geographical differences remains difficult due to different study methodologies (Stacy and Innis 2017).

When reviewing haematology, it was evident that PCV was similar across WA rookeries collected during the summer nesting season, but higher than samples collected from nesting flatback turtles in NT and Queensland rookeries in the winter nesting season. Haemoglobin values for nesting turtles were very similar to previously reported values in summer nesting flatback turtles in Queensland (Sperling et al. 2007).

Similar to Scheelings et al. (2020), heterophils were the predominant WBC in nesting turtles, followed by lymphocytes. White blood cell differential and absolute counts were relatively similar to nesting flatback turtles in Scheelings et al. (2020), except for slightly lower lymphocytes and higher monocyte counts. Our nesting H: L ratio values were comparable to Scheelings et al. (2020), with higher and lower limits.

There were significant regional differences for electrolytes when compared to other flatback turtle populations. Bare Sand Island flatback turtle sodium and magnesium values were significantly lower, and chloride to a lesser degree (Guinea 2016), than values in this study. These lower electrolyte values could be due to geographical reasons, including lower water salinity at higher latitudes (southern hemisphere), and subsequent osmoregulatory differences. However, it could also be due to a myriad of other factors including other environmental conditions such as increased local winter water temperatures (Bureau of Meteorology 2013, 2014, ABC News 2018) (noting this is a winter nesting rookery), different sizes (NT study turtles were smaller) or delayed plasma separation of 24 hours (Dr M. Guinea personal communication), the latter is especially applicable to sodium (Abou-Madi and Jacobson 2003, Eatwell 2007). Scheelings et al. (2020), reported extremely high potassium values (average 7.2 mmol/L). While comparatively high reports of potassium

have been reported in foraging loggerhead turtles in Deem et al. (2009), such high results are unexpected, with values >5.5 mmol/L associated with mortality (Stacy et al. 2013), and especially due to the proximity of one site to Eighty Mile Beach (Cemetery Beach, Port Hedland). However, with sampling earlier in the season at Cemetery Beach, potassium is expected to be higher due to more recent feeding (Stamper et al. 2005, Deem et al. 2009, Honarvar et al. 2011).

Potassium is one of the analytes most frequently impacted by haemolysis and other artefacts. Generally, potassium and phosphorus (and AST) are reported to increase with haemolysis, increased time to separation and increased storage temperatures as intracellular potassium is released from damaged erythrocytes, while reports of sodium and chloride being more variable, were usually reported to decrease in other species (Christopher et al. 1999, Abou-Madi and Jacobson 2003, Eatwell 2007). However, a recent publication in sea turtles found all electrolytes increased with haemolysis (Stacy et al. 2019b). Scheelings et al. (2020) study, also reportedly used serum. Generally, lithium heparin is the preferred anticoagulant of choice in sea turtles and serum is not used for a number of reasons including the unpredictable clot formation, and for the occurrence of potential 'pseudohyperkalaemia' from thrombocyte potassium release in the clot (Bolten et al. 1992, Raskin 2000, Campbell 2012, Stacy et al. 2019b).

3.4.7 Comparisons with other species

Nesting PCV values for flatback turtles in the present study were higher than nesting WA loggerhead turtles but fell within range of other loggerhead (Casal et al. 2009) and green (Samour et al. 1998) nesting turtle populations, with similar mean values to olive ridleys, hawksbills, and leatherbacks (Alkindi et al. 2001a, Honarvar et al. 2011). Foraging flatback PCV values were similar to some species, including green, loggerhead, olive ridley, and hawksbill turtles (Day et al. 2007, Komoroske et al. 2011, Muñoz-Pérez et al. 2017, Espinoza-Romo et al. 2018), less than leatherbacks (Innis et al. 2010) and higher than other olive ridley populations (Reséndiz et al. 2019). As expected, adult foraging flatbacks generally had higher PCV values than studies which included subadults and juveniles (Deem et al. 2009) as PCV has been shown to be positively correlated to body size (Casal et al. 2009, Basile et al. 2012, Kelly et al. 2015, Stacy et al. 2018a).

When compared to other nesting turtles, flatback Hb values were lower than leatherbacks (Stacy et al. 2019b), higher than nesting olive ridleys (Alkindi et al. 2001a, Santoro and Meneses 2007), loggerheads and greens (Alkindi et al. 2001b, Alkindi and Mahmoud 2002), but similar to hawksbills (Alkindi et al. 2001a). For foraging individuals, flatback values were similar to adult loggerheads (Basile et al. 2012), and higher than olive ridleys (Reséndiz et al. 2019) and greens (Samour et al. 1998).

As with other species, foraging flatback RBC values were higher than nesting flatback values (Harris et al. 2011), and as with other haematological values, flatback RBC values were less than leatherbacks (Harris et al. 2011), and similar to foraging loggerheads (Basile et al. 2012) and more than foraging olive ridleys (Reséndiz et al. 2019). Overall flatback PCV, Hb and RBC values were higher than most other species, except leatherback turtles, which is likely related to their longer dives and probable 'higher oxygen carrying capacity' (Lutcavage and Lutz 1997, Sperling et al. 2007, Stacy et al. 2019b).

As for remaining calculated blood parameters (MCHC, MCH, MCV), these fell within ranges for foraging green turtles (Samour et al. 1998), but were higher than foraging olive ridley turtles (Reséndiz et al. 2019). For nesting turtles, values were lower than green nesters (Lara-Uc et al. 2016), while the MCHC was similar to hawksbill turtles, higher than loggerhead (Alkindi and Mahmoud 2002) and olive ridley turtles (Alkindi et al. 2001a), but had a wider range than other olive ridley populations (Santoro and Meneses 2007) and lower than another green turtle nesting population (Alkindi and Mahmoud 2002).

Our WBC parameters were generally lower than nesting WA loggerhead turtle values (Trocini 2013), which also had heterophils as the predominant WBC in nesting turtles, followed by lymphocytes. Our nesting H: L ratio values were in disagreement to Harris et al. (2011) findings in leatherbacks. Heterophil: lymphocyte ratio has been utilised as a proxy for stress in animals, including reptiles (Davis et al. 2008) and further for disease assessment in sea turtles (Aguirre et al. 1995, Work et al. 2001, Work et al. 2003). Similar to other studies by Innis et al. (2014) and Flower et al. (2018), we found positive correlations between H: L ratios and potentially 'stressful' scenarios, including nesting time-period, time restrained, and number of blood sampling events. This relationship was found in nesting turtles only, possibly related to existing lower heterophil levels, as well as winter sampling of foraging

turtles and suppression of immune response (Zapata et al. 1992). Guinea (2016) attempted to assess restraint effects on biochemical parameters to infer levels of stress in flatback turtles, and while this warrants investigation, inclusion of H: L ratio would have been worthwhile. Unfortunately, too few animals were sampled without restraint in our study to assess the effect of the restraint method.

Basophils are rare in sea turtles, and only low numbers were detected in this study with levels comparable to other species. Little is understood about basophils in sea turtles (Stacy et al. 2011), however, like with other species, one potential cause for increased numbers could be related to haemoparasites (Rosskopf Jr 2000). Basophils were only present in a small number of turtles (n=23; foraging 13/49, 26.5%; nesting 10/146, 6.8%), many of which were removed as outliers, especially for nesting turtles. However, finding potential haemoparasitic vacuoles were 3.6 times higher (OR=8.40, 95% CI: 3.2-26.6) in turtles with basophils warrants further exploration. While eosinophilia is frequently reported with parasitism, our study found no relationship between eosinophils and these vacuoles.

These flatback RRs generally fell within RRs for comparative species i.e., nesting turtles and adult foraging turtles (Bolten and Bjorndal 1992, Hasbún et al. 1998, Deem et al. 2006, Hamann et al. 2006, Santoro and Meneses 2007, Casal et al. 2009, Deem et al. 2009, Flint et al. 2010b, Innis et al. 2010, Harris et al. 2011, Perrault et al. 2012, Trocini 2013, Whiting et al. 2014b, Guinea 2016, Sözbilen and Kaska 2018, Scheelings et al. 2020) except cholesterol and triglyceride were particularly high for both nesting and foraging flatback turtles, with a higher upper limit than other ranges reported. Nesting flatback turtles had a lower ALT, bilirubin T, and phosphorus as well as higher magnesium, while foraging adult flatbacks also had low glucose (Fl-Ox) and iron, with the lower iron limit only comparable to juveniles (Whiting et al. 2007a, Whiting et al. 2014a, Whiting et al. 2014b). However, it should be noted plasma iron is not an accurate representation of overall body iron status (Stacy and Innis 2017). Apart from GLDH where no RVs were available for sea turtles, with values within range for tortoise spp. observed by Scope et al. (2013), all other analytes were within range of sea turtle species.

The only RRs the higher magnesium limit in both nesting and foraging animals fell within, along with the lower glucose (Fl-Ox) limit (foraging individuals only), were those of Hamann

et al. (2006), which were reportedly outside other RRs for these two analytes. The low glucose values in our study were likely artefactual, as the author was advised to use fluoride oxalate and not to separate the plasma (by the laboratory), similarly Hamann et al. (2006) used sodium citrate, known to reduce glucose results and increase haemolysis (Phillips et al. 2017). Plasma magnesium is reported to increase in mammals with haemolysis, and when exposed to higher temperatures and when time to analysis is prolonged, due to the high intracellular magnesium content (Thoresen et al. 1992, Stacy et al. 2019b). The magnesium reading on the Beckman Coulter AU680 biochemistry machine used in our study, is reportedly more affected by haemolysis interference than other analytes (Beckman Coulter 2009). However, in our study and Hamann et al. (2006), both reported the exclusion of haemolysed samples. While the reason for the increased magnesium remains unclear, it remains highly possible the values are higher due to physiological, nutritional, osmoregulatory or other environmental reasons (Lutz 1997, Whiting et al. 2007a, Innis et al. 2017b, Bloodgood et al. 2019). Finally, cholesterol was even higher than reported in leatherback turtles, and interestingly, higher cholesterol was related to lower hatching and emergence success rates compared to successful rates in leatherbacks (Perrault et al. 2012).

3.4.8 Novel intraerythrocytic haemoparasite

During this study, a novel intraerythocytic haemoprotozoan was detected through morphological and molecular testing in flatback turtles (and juvenile green turtles, data not shown). Initially disregarded as artefactual, closer examination (including electron microscopy) revealed ultrastructural details of the haemoparasite life stages with preliminary molecular studies placing it as closely related to parasites from the Bellingen River snapping turtle (*Myuchelys georgesi*) *Haemocystidium* sp. and *Haemoproteus* sp. from the Greek tortoise (*Testudo graeca*) (Orkun and Güven 2013). Trocini (2013) first discovered protozoal haemoparasites, similar to *Haemoproteus* (found in freshwater chelonians – now known as *Haemocystidium*), during her PhD research on WA loggerhead turtles. Her study had detection rates of 3.1% (n=161) and no haemoprotozoal infection in foraging loggerhead turtles (n=72). Although haemoparasites were detected in only one turtle in this study (0.5%; 1/195), intraerythrocytic vacuoles (which may be related to the presence of a haemoparasite, such as a potential life stage of the parasite) were detected in 17.8% of

nesting flatback turtles and 100% of flatbacks at the Roebuck Bay foraging site. Given malarial parasites are not thought to exist in sea turtles (Stacy and Innis 2017), or other marine reptiles (Trocini 2013), and reportedly intraerythrocytic protozoal vacuoles have previously been disputed due to mis-identification or lack of evidence (Stacy and Harvey 2015), further work is required to determine PCR prevalence, and importantly the relationship of the presence of these haemoparasites to health.

3.4.9 Field vs laboratory results

While an important necessity, our results comparing field and laboratory testing show significant incongruencies. With the i-STAT consistently measuring lower than the laboratory values, despite the time lag to laboratory testing (i.e., i-STAT field test <8 hours, laboratory testing <60 hours).

While most correlations appeared good to excellent, there were proportional or constant biases detected with Passing-Bablok or Bland-Altman analysis for the majority of the analytes, with PCV probably the only exception, where during the early data clean-up phase, differences of >20% for laboratory and field PCV were excluded. While Bland-Altman provides the limits of agreement for the different methods (mean +/- 2SD) (Dewitte et al. 2002), whether these are clinically acceptable limits must be determined by the clinician (Giavarina 2015). For example, comparing total protein and TPS, the refractometer reading is affected by non-protein solutes such as BUN, glucose, cholesterol, lipaemia (Bolten et al. 1992, Fudge 2000b), and while it is only an estimate, has a constant bias of +10 and a negative proportional bias, with differences of up to 35 g/L and is therefore not clinically acceptable. Aside from i-STAT HCT, the other comparisons were relatively interchangeable once constant bias has been taken into consideration. This further strengthens the argument different RIs should be developed for different analysers (McCain et al. 2010), but the need for practical clinical testing in the field remains an important area to investigate.

3.4.10 Limitations

Field work in remote locations presents multiple challenges. These include the logistics of collection, processing and transportation along with the unfortunate event of temporal and

temperature effects on samples causing artefact and rendering samples null and void. Working in the field is highly variable and non-comparable to laboratory conditions. Furthermore, extreme weather conditions (e.g., heat, rain, cyclones, wind) can also present limitations such as the inability to make fresh blood films in the field. While there is no gold standard for reptilian haematology (Winter et al. 2019), ideally, Natt-Herricks method (Natt and Herrick 1952) along with blood film estimate should be used for white blood cells counts, but unfortunately the former method was not available at the commercial laboratory, nor was it available to the researcher in the field (Sheldon et al. 2016, Stacy and Innis 2017). But as Stacy et al. (2019b) notes, many studies still use the WBC estimation technique. Once samples were at the laboratory, although a single clinical pathologist was requested to analyse the samples, multiple worked on the cases, and any inter-observer variation (such as haemolysis grading of plasma samples) was not accounted for.

A major challenge with comparing results to other studies is the use of different methodologies potentially resulting in different values such as different analysers (McCain et al. 2010), anti-coagulants (Hrubec et al. 2002) and plasma storage conditions, for example fresh compared to frozen (Ramer et al. 1995, Hawkins et al. 2006). The use of different haematology analysers for nesting flatback turtles (Cell-Dyn 3700) and foraging flatback turtles (Sysmex XN-1000) in this study, has been identified as a limitation for the comparison of haematological values in the different life stages. Additionally, many studies lack adequate reporting of methods and use inappropriate statistical testing and have not considered the significant impact of artefact. Artefacts, such as haemolysis, are a serious problem that affect the reliability of results and while every effort was made to minimise artefacts, in some circumstances haemolysis was unavoidable. Even mild haemolysis can have an impact, and this frequently goes unreported (Stacy et al. 2019b).

3.4.11 Recommendations

When developing RIs, it is absolutely paramount to adhere to the IFCC-ASCVP guidelines starting with the experimental design, using rigorous methodology, and undertaking appropriate statistical analyses to create robust RIs to avoid pre-analytical, analytical or post-analytical artefacts, with the overall aim to strive for consistency. As the first RIs for (nesting) flatback turtles, these results indicate an increased sample size namely juveniles,

foraging males, foraging turtles of known reproductive status, and nesting females at the end of the season would provide clearer insight into different boundary groups, as would sampling breeding males. With continued collection of satellite tracking data, we can start to examine health data for flatback turtles from the same home-ranges, and turtles preparing for breeding migrations (pre-breeding). Additionally, the use of ultrasound to assess reproductive status and association with blood values would be useful. The remote locations of much of the fieldwork resulted in increased in time to analysis (increased from 48 to 60 hours, and centrifugation from six hours to eight hours). While this represents the reality of working in these locations, further investigation is warranted to accurately assess whether these extended timeframes significantly affect haematology and biochemistry results. A larger sample size to reduce risk of type II error (false negative) is also recommended. Finally, ongoing research into novel biomarkers indicative of health status such as haptoglobin and hepcidin is needed. Further investigation of the association between the novel intraerythrocytic parasite and clinical disease has also commenced.

As the first RIs for flatback turtles, these results indicate they can be used as an alternative for the different boundary groups, especially when taking into consideration statistical differences which may not be clinically significant. For example, although only 8/44 analytes were similar for life stage (36/44 analytes, $p<0.05$), an additional six analytes had differences not deemed to be clinically significant, such as difference of only 1.4 mmol/L (0.9%) for sodium ($p=0.038$). Focusing on the most useful analytes in assessing health (including RBC and WBC parameters, CK, AST, BUN, uric acid, glucose, sodium, potassium, chloride, protein, calcium, and phosphorus), these RIs can be used for:

- Other flatback rookeries excluding BUN, potassium, chloride, protein (taking season into consideration);
- Pre-breeding foraging female flatbacks excluding haematological parameters, BUN, glucose, sodium, potassium, and protein;
- Foraging female flatbacks excluding haematological parameters, CK, BUN, glucose, potassium, chloride, and phosphorus.

Due to the significant number of statistical differences, not to mention clinically significant differences, it would be recommended these RIs are not used for haematology for mature males or immature foraging flatbacks, and only judiciously for biochemistry (i.e., sodium and protein). If available, these RIs should be used in conjunction with the descriptive statistics

tables and supplementary tables in the Appendices appropriate to the boundary condition under investigation. Notwithstanding, differences between study methodologies should always be considered prior to comparing RIs.

In the absence of other RIs and until further sampling can be performed for the various boundary conditions, these RIs have filled a major knowledge deficit for flatback turtles plus they are more representative of the species, covering multiple locations, over multiple seasons of mixed genetic stocks, but from one life stage (nesting).

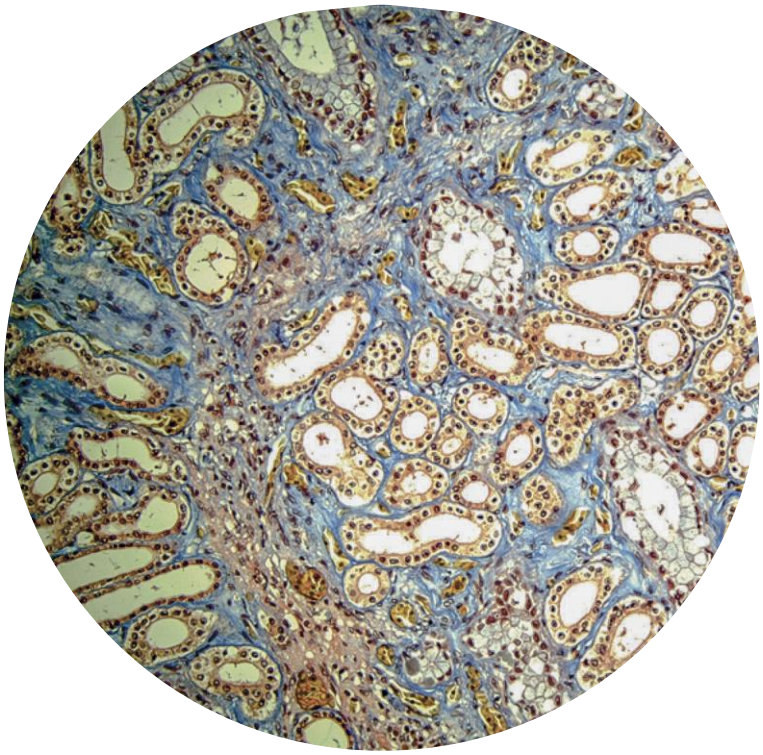
3.4.12 Conclusions

This research has contributed to knowledge regarding the health of the 'data deficient' flatback turtle, providing insight into flatback blood RVs and completing RIs for the last sea turtle species, for which such values were unknown (Stacy and Innis 2017), as well as discovering a novel malarial intraerythrocytic parasite.

This data show moderate variability in flatback turtle RIs, and after acknowledging methodological differences exist, overall results were comparable to other flatback turtle and other sea turtle species results, with similar differences for the various boundary conditions. Sea turtles regulate their blood values over wide ranges and considering these can vary seasonally, even with these boundary group differences, it is questionable how useful or practical creating separate RIs for individual sites/different populations is. Serving as a proxy for WA flatback turtle populations, analysis of the flatback turtle health assessments from three sites (Eighty Mile Beach, Thevenard Island and Roebuck Bay), provides an early indication WA flatback turtles are in good health. As indicators of environmental health (Aguirre and Lutz 2004), correspondingly this would also indicate their habitat is also healthy.

In a changing world, establishment of wildlife health baseline data are of paramount importance to monitor health status and secure the survival of wildlife populations. As was found in this study, there are many challenges associated with working with wildlife, often in remote locations with highly variable collection conditions. Often limited sample sizes are responsible for wildlife RI studies failing to meet recommendations for RIs and partitioning, which was fortunately not the case in this study. Additionally, outlier detection and scrutiny

is particularly critical when convenience sampling is used, which is frequently the case with wildlife (Friedrichs et al. 2012). Overall, the best way to develop robust RIs is to ensure standardisation of methodology and rigorous adherence to guidelines, as well as collection of samples from a healthy population (Friedrichs et al. 2012). This is often a challenge for wildlife studies with largely unknown histories. Notwithstanding these factors, these comprehensive results will be invaluable for assessing, and monitoring clinical cases in rehabilitation, monitoring trends of wild populations, and for future health-related research.



CHAPTER 4

Morbidity and mortality investigations of stranded sea turtles in Western Australia

4.1 Introduction

Western Australia has six marine turtle species in its waters; the green, loggerhead, hawksbill, olive ridley, leatherback and endemic flatback turtle, with nesting populations for all species except the leatherback. All six species are listed as threatened and protected fauna under WA's *Biodiversity and Conservation Act 2016.* Within the state, the flatback, green, hawksbill and leatherback turtle are listed as 'vulnerable', and the loggerhead and olive ridley turtle are listed as 'endangered'. Many global threats to sea turtles also threaten WA sea turtle populations, and the majority of these are anthropogenic (Commonwealth of Australia 2017b, Witherington 2017).

The Australian Marine Turtle Recovery Plan (Commonwealth of Australia 2017b) has identified common threats across many of the genetic or RMU stocks which include climate change, pollution, development (primarily from industry) and light pollution regarded as 'high' risk for most WA populations. The international illegal harvest of WA hawksbill turtles is classified as 'very high' risk, and diseases and pathogens are rated as 'low' risk for WA populations, despite >85% of this threat category concurrently classified as 'unknown' risk. Similar levels of uncertainty regarding the risk of disease to sea turtles were reported in other parts of Australia, predominantly due to the lack of stranded sea turtle disease investigations nationwide.

A 'stranded' sea turtle refers to any live or dead sea turtle found floating or washed up along the coastline, including tidal water inlets, presenting with injuries, disease or other abnormalities, which includes behavioural abnormalities in live turtles (Stacy et al. 2017b). Sea turtles strand for a variety of reasons, including trauma (anthropogenic and natural), chronic debilitation, interaction with marine debris, interaction with fisheries, buoyancy disorders, cold-stunning, as well as from toxicities and other infectious and non-infectious diseases. Although much information can be gleaned from external examination and circumstantial evidence, causes of stranding (and mortality) derived from this information alone are generally non-specific, and tend to include syndromes such as chronic debilitation and buoyancy disorders. Consequently, the specific causes of mortality cannot be determined without detailed veterinary diagnostic and pathological investigations.

Disease investigations of stranded sea turtles involve a variety of ante-mortem and post-mortem veterinary diagnostic testing to understand the causes of lesions, disease and other debilitation, and mortality. Necropsy is the most effective diagnostic technique to investigate mortality, as it is low cost and requires minimal use of technology (Gordon 2005, Work et al. 2015). Ancillary testing, such as clinical pathology, diagnostic imaging, parasitology, microbiology and histopathology, as well as other molecular techniques, also play an important role in reaching a definitive diagnosis. Stranded sea turtles are usually found deceased in various states of decomposition (Chaloupka et al. 2008, Barco et al. 2016). Therefore, those found alive are of high value in morbidity and mortality studies. These individuals have a higher chance of definitive diagnosis, for a number of reasons, including: the relatively high-quality of samples that can be obtained from fresh or freshly frozen specimens; known clinical histories for individuals that enter rehabilitation after stranding (this is important, given that clinical histories are usually unknown for wildlife); and the potential to use ante-mortem findings in conjunction with post-mortem results to assist in reaching a diagnosis. For example, deficits detected on neurological examination can help to localise the specific anatomic position of a lesion; and in conjunction with pathological findings, can allow a better understanding of disease processes contributing to mortality (Chrisman et al. 1997, Jacobson 2006). Aside from establishing cause of death, disease investigations are also useful for identifying background pathology in healthy animals (e.g., in acute trauma cases), establishing baselines to monitor disease trends, and discovering potentially novel and emerging diseases (Flint 2013, Stacy et al. 2017a). It should be noted that although necropsies are a biased subsample of the population (i.e., only sick turtles that died and were found stranded vs those that may have disappeared or those that may have recovered) and live turtle investigations help address this bias, there are still limitations associated with ante-mortem investigations.

Causes of mortality can be classified into two broad categories: ‘natural’ (e.g., infectious disease and non-infectious disease), and ‘anthropogenic’ (including both direct and indirect anthropogenic factors). While direct anthropogenic threats, such as vessel strike and entanglement, are often readily apparent, indirect anthropogenic threats are often insidious and can overlap with ‘natural’ disease (Stacy et al. 2017a). Indirect anthropogenic threats include environmental changes which can affect host-pathogen relationships and can lead

to subclinical disease (which can still affect fitness, reproduction and survival), through complex pathways and multifactorial processes (Deem et al. 2001, Commonwealth of Australia 2017b). Anthropogenic threats are generally regarded as the primary cause of sea turtle mortalities, and there is increasing recognition not only of the importance of 'natural' disease (both infectious and non-infectious) in mortality (Deem et al. 2001, Hamann et al. 2010, Flint et al. 2015b), but of the major role that indirect anthropogenic threats play in disease expression (Stacy et al. 2017a).

In some cases the causes of sea turtle stranding and mortality appear obvious (Foley et al. 2019) – for example a turtle in good body condition with acute traumatic injuries consistent with vessel propeller strike (Barco et al. 2016). However, disease investigations are still important for supporting or refuting initial hypotheses, and for identifying any co-existing morbidities or other variables that may predispose to disease or injury (Gordon 2005). For example, histopathology can help to determine whether observed amputations occurred pre- or post-mortem (e.g., through scavenging), or if an infection secondary to trauma contributed to death through bacterial septicaemia (Flint et al. 2009b); and, likewise whether a pre-existing condition may have caused the individual to float, predisposing it to a higher risk of vessel-related trauma (Commonwealth of Australia 2017b, Mettee and Norton 2017).

Although disease is generally associated with infectious agents, such as parasites and viruses, disease can be associated with any conditions that contribute to ill-health. Wobeser (1981) defines 'disease' as "any impairment that interferes with or modifies the performance of natural function". In our study, we extended this definition to include non-infectious aetiologies such as toxins, interaction with marine debris and trauma (Deem et al. 2001, Wobeser 2006, Deem and Harris 2017); many of which are important disease threats with anthropogenic origins. Despite recent advances, our understanding of sea turtle disease remains limited (Flint 2013), including 'natural' diseases and potential associations with anthropogenic activities. For example, despite large research efforts to identify the aetiology of the globally significant fibropapillomatosis tumour disease, and the implication of a chelonian herpesvirus in the disease, the specific cause of fibropapillomatosis remains uncertain (Herbst 1994, Jones et al. 2016).

Another sea turtle disease of importance of which much remains unknown, is spirorchiidiasis (Chapman et al. 2019). Spirorchiidiasis is a very common, widespread disease of sea turtles globally, with a prevalence of up to 98% in some Australian sea turtle populations (Gordon et al. 1998a). The specific disease agents are spirorchiid blood flukes, of which there are currently 29 known species — although this number is predicted to increase when further research is undertaken in understudied regions and sea turtle species, and with advances in molecular technology (Stacy et al. 2010a, Chapman et al. 2019). Spirorchiidiasis is primarily a disease of the cardiovascular system; the adult parasites can induce severe pathology in the major vessels of sea turtles, including aneurysms, thrombi and vasculitis (Stacy et al. 2017c). The adult spirorchiids are fecund, producing thousands of eggs which travel throughout the circulatory system lodging in small vessels, which commonly includes the GI tract, lungs and spleen (Ladds 2009). These eggs can incite significant inflammatory host reactions, forming granulomas comprising mostly mononuclear cells, often featuring multi-nucleated giant cells, and occasional granulocytes and fibrosis (Gordon 2005, Ladds 2009).

The life cycle of these spirorchiid parasites remains largely unknown (Chapman et al. 2019); and has been extrapolated from freshwater species (Figure 1.7). It is believed that the spirorchiid egg hatches in the environment, and that the miracidia – motile free-living larvae – enter an intermediate host (Stacy et al. 2017c). This life stage has only ever been found in three intermediate host species: a limpet, worm snail and a polychaete worm (Stacy et al. 2010b, Cribb et al. 2017, de Buron et al. 2018). It is then thought that fork-tailed cercariae emerge from the intermediate host and enter the definitive host – the sea turtle – probably by penetrating mucous membranes, such as the conjunctiva (Stacy et al. 2017c). These cercariae develop into adult spirorchiids in large vessels. Eggs are deposited in the blood stream by the adult parasites, and either remain at the primary site of oviposition (identified by presence of adults and/or egg masses >100 eggs) or embolise to secondary sites in various organs (<20 eggs and disseminated distribution) (Stacy et al. 2010a). It is not known exactly how the eggs re-enter the environment; it is hypothesised that this may occur either through expectoration or defecation, or potentially through scavenging on sea turtle cadavers (Chapman et al. 2019). A major challenge with this disease is the attribution of significance, given that sea turtles which present in seemingly good health can have spirorchiid infection, with severe pathology (Santoro et al. 2006). This supports the idea that spirorchiids are not

only opportunistic pathogens in debilitated animals, but may also function as primary pathogens (Stacy 2008). If this is the case, it is not clear under what conditions subclinical infection occurs; although it is hypothesised that there may be a cumulative effect, where disease occurs above a certain threshold of parasite intensity (Gordon 2005, Stacy et al. 2010a).

An essential first step towards understanding the current health status and health threats of sea turtle populations is establishing baselines for morbidity and mortality through investigations such as stranding surveys. Stranding surveys can also serve many other purposes, including: a proxy for sea turtle population distribution and abundance (Chaloupka et al. 2008); a form of passive population health surveillance; identification of unusual mortality events; detection of anthropogenic mortalities; and subsequently determining whether these mortality rates are sustainable for population viability. For example, in some areas the most serious threat to population viability is watercraft- or fisheries-related mortality of breeding turtles with the breeding turtle life stage being the most reproductively important life stage (Heppell et al. 2003, National Research Council (NRC) 2010, Foley et al. 2019). Understanding these current and future stressors on sea turtle health has clear conservation implications, and as such, investigating causes of morbidity and mortality is critical for policy makers managing sea turtle populations.

To date, there are a lack of baseline data on the causes of morbidity and mortality in WA sea turtles. Only sporadic, isolated case reports of disease in green and loggerhead turtles exist. Such reports include two clinico-pathological disease investigations from WA; including one study which reported spirorchiidiasis and associated Gram-negative bacteria in four green turtles from Exmouth, Karratha and Monkey Mia (Raidal et al. 1998), and another study reporting osteoarthritis and spirorchiidiasis in a loggerhead from Perth (Raidal et al. 2006). Other health studies for sea turtles in WA include investigation of marine debris ingestion in green and loggerhead turtles from Ningaloo and Shark Bay (Reinhold 2015) and arsenic levels in a leatherback from Cervantes (Edmonds et al. 1994). Several studies of GI parasites in sea turtles in Shark Bay investigated nematodes (Lester et al. 1980) and digenean trematodes (in loggerheads only) (Blair and Limpus 1982). Fibropapillomatosis was first

confirmed histologically in a WA study in a green turtle from Shark Bay with tumours (Raidal and Prince 1996). Prince et al. (2012) reported further FP cases in WA from the Exmouth Gulf. Additional histological descriptions of FP biopsies from two nesting loggerhead turtles from Dirk Hartog, WA were reported by Trocini (2013). Stranding data recorded in WAStD (accessed 21 April 2021), along with other stranding reports on leatherback, flatback and hawksbill turtles (Prince and Crane 1996, Prince 2004, 2007) have also contributed to our knowledge of stranded sea turtles in WA.

Although there is a high volume of research on specific sea turtle diseases (including FP) in other parts of Australia, especially Queensland, the major sea turtle morbidity and mortality studies involve necropsy and pathology of wild green turtles (Glazebrook et al. 1989, Glazebrook and Campbell 1990b, Gordon et al. 1998a, Gordon 2005, Flint et al. 2010d, Flint et al. 2015b); alongside a small number of loggerhead and hawksbill turtles examinations (Glazebrook et al. 1989, Glazebrook and Campbell 1990b), and a few studies of diseases in captive sea turtles (Glazebrook and Campbell 1990a, b). However, there is a paucity of information regarding the disease status of flatback and olive ridley turtles in Australia.

Globally, causes of death in stranded sea turtles remain largely unknown; with most studies recording details pertaining to the stranding event (such as observed external abnormalities and stranding-related temporo-spatial data). There is minimal information focused specifically on cause of death (Chaloupka et al. 2008, Casale et al. 2010, Stacy et al. 2017a). Furthermore, with the exception of reported FP in a flatback turtle (unconfirmed histologically) (Limpus et al. 1993), there is a lack of disease reporting in flatback turtles, despite their large endemic populations in WA and other parts of northern Australia (Stacy et al. 2017c, Chapman et al. 2019). Few studies have correlated clinical findings with pathology (Gordon 2005, Flint et al. 2010d, Oros et al. 2016); as this generally requires veterinary expertise (Stacy et al. 2017a). Historically, most work on sea turtle strandings has instead been performed by biologists without formal training in veterinary necropsy procedures and with a different focus when performing necropsies, for example physiology and reproduction.

Human-induced environmental change continues to place pressure on the health and survival of sea turtles. Therefore, investigating causes of morbidity and mortality of stranded

turtles is integral to monitoring sea turtle health, to identify new and emerging diseases and, to understand the impact of diseases and pathogens. As sea turtles are regarded as a sentinel species (Aguirre and Lutz 2004), such studies can also be used to monitor environmental health. This is the 'One Health' approach; examining the relationships between human, environmental and animal health and acknowledging that all three are interconnected and dependent on the health of each other (Daszak et al. 2000). As diseases in the marine environment continue to increase, disease monitoring is also essential for prompt identification of emerging threats (Harvell et al. 2002).

Establishing causes of stranding and mortality of sea turtles is not without its challenges. Reaching a final diagnosis can be difficult; for example, in cases where turtles have chronic illness involving multifactorial disease (Stacy et al. 2017a), or in cases where already autolysed cadavers have been frozen then thawed (Dobbs 2001, Chaloupka et al. 2008, Cook et al. 2020). These issues, alongside a lack of history, are common barriers to the identification of causes of stranding and mortality in wildlife studies (Stacy et al. 2017d).

The vastness of WA combined with the remoteness of much of the coastline mean researchers are particularly challenged by the issues outlined above. Many stranded turtles are found in decomposed conditions, especially in the remote and northern parts of the state, which experience the hottest weather. To preserve stranded sea turtle cadavers in warmer conditions in regional parts of the state, the cadavers are typically stored in freezers. While freezing is preferred to disposal and a missed diagnosis, freezing can cause artefacts and obscure subtle pathology (McAloose et al. 2018), especially in an already autolysed cadaver.

Tens of sea turtles are reported stranded around the WA coastline each year, but it is likely hundreds could go unreported due to the remoteness of much of the state (Dr S. Whiting, DBCA, personal communication, May 2016) (Raidal et al. 2006). This is similar to other areas where reported stranded sea turtles are a small percentage of stranding mortalities (Epperly et al. 1996, Hamann et al. 2010, Barco et al. 2016, Stacy et al. 2017b). While necropsy may have been performed, to date the cause of death has only been documented in a handful of stranded sea turtle cases in WA. With the focus of our study on causes of mortality, only ante-mortem and post-mortem results from deceased turtles are presented in this chapter.

Given the specific data selected for use, the aim of this study was to investigate the causes of morbidity and mortality of sea turtles in WA primarily through necropsy, histopathological and parasitological examination. Where possible, other ancillary diagnostic tests were also utilised. We examined variables such as age-class, sex, region and season as risk factors for disease, with a specific focus on spirorchiidiasis.

The objectives of the study were to:

(i) Establish a benchmark for primary causes of morbidity and mortality of sea turtles in WA, and determine if the diseases are similar to those affecting turtles elsewhere.

(ii) Determine whether, for stranded sea turtles, natural causes of morbidity and mortality are more commonly observed than anthropogenic causes; and compare these findings to other studies.

(iii) Determine whether spirorchiids infecting turtles in WA are the same species and genera as those reported in other studies, and if the effects on the host are similar.

(iv) Investigate the incidence and intensity of spirorchiidiasis; assess the significance of infection; and compare these findings with other studies. Additionally, to compare the usefulness of the different methodologies used in diagnosis.

(v) Investigate the association between the various risk factors (for example species, age-class, sex, region, season) and the manifestation of different diseases or syndromes, with a particular focus on spirorchiid-related health observations.

(vi) Investigate any correlations between ante-mortem and post-mortem findings.

(vii) Demonstrate the value of detailed veterinary diagnostic testing in reaching a final diagnosis for cause of death, and compare benefits of the different methodologies.

4.2 Methods

4.2.1 Study animals

The study animals included stranded sea turtles from WA which had a necropsy examination performed by the author (n=75). All stranded turtles were found stranded during the study period between 2016 – 2018, except for three turtles which stranded in either 2014 or 2015.

Prior to, and in the early stages of the study, canvassing was performed to inform relevant stakeholders (e.g., PZVD, DBCA metropolitan region and regional Marine Parks) of the study's intent and to request notification regarding stranded sea turtles. Selection of stranded sea turtles for necropsy in this study was opportunistic. Any stranded turtle that was reported was a potential candidate, provided that the stranded turtle was retrieved, transported and/or frozen, and (in the case of cadavers) in a suitable condition for necropsy examination.

The agency responsible for stranded sea turtles and maintaining the WAStD for stranded sea turtles, is DBCA. When a turtle strands in WA, a marine turtle stranding form is generally completed, which includes pertinent data such as date, location, status (dead or alive), decomposition state of the cadaver, any obvious external abnormalities (e.g., fibropapilloma tumours, amputations or other damage/injuries), flipper tag details (if present), various morphometrics (CCL, CCW, *CT*, HW) as well as species, sex and age (if known), and any other useful circumstantial information (Appendix 2 DBCA Marine Turtle Stranding Form, 2018). These data, along with any photographs, are subsequently entered into WAStD. For this study, WAStD was searched to compare numbers of stranded turtles reported across WA with the number included in this study, and to confirm whether a stranding record of the turtle existed, and that data in the database correlated with data collected during the detailed veterinary disease investigations.

The potential effect of region on stranded turtle reporting frequency was investigated by dividing the state into north and south. The northern extent of the Mid-West District (DBCA designated District) at Kalbarri (27.7105°S, 114.1651°E) was used as the boundary, due to the different climatic conditions between the lower latitudes (tropical climate) and the higher latitudes (Mediterranean climate). Timing of stranding frequency was also examined with respect to relative temperature of the ocean in different seasons, with winter and spring classed as 'cool', and summer and autumn as 'warm' (categorical variables). Investigations for differences in stranding frequency were also examined by season in each of the different regions: the north region (wet summer, dry winter) and the south region (wet winter, dry summer). For the north, the seasons were divided into wet and dry seasons, with the wet season assigned as November through April, and the dry season as May to

October. The month of stranding was also selected as a variable of interest, to examine the potential effect on stranding frequency.

4.2.2 Live turtles

The live stranded turtles included in this study comprised only those which died or were euthanased, and subsequently underwent necropsy examination.

Rehabilitation

Rehabilitation cases included live stranded turtles which entered care at either veterinary clinics or rehabilitation facilities with wildlife rehabilitators, and were not euthanased at the time of arrival (either before or after an initial assessment on arrival). Live cases which died before arriving in care or were euthanased in the field were excluded from this group. For turtles rehabilitated at multiple facilities, the facility reported was the facility at which the turtle spent the most time. Time in care was categorised by duration into short (<7 days), medium (7-28 days) and long term (>28 days).

Veterinary clinical examination

Two veterinary assessments were performed on sea turtles admitted to PZVD (+/- regional veterinary clinics) by the attending veterinarian. An initial veterinary assessment was performed on arrival, followed by a more extensive standardised sea turtle diagnostic examination, usually on day three but occasionally up to day five for logistical reasons. The main purpose of the initial assessment was to gather basic health data, triage, address any critical health issues and stabilise the patient. The standardised diagnostic examination provided an opportunity to conduct a thorough health assessment, including further testing.

The standardised diagnostic examination assessed weight, body condition score (BCS), hydration status, mentation, activity, any external or behavioural abnormalities (such as injuries, heavy epibiota or neurological deficits), ocular ulceration, cloacal temperature and various morphometrics (CCL, CCW, HW and *CT*). Buoyancy, appetite and defecation were also reported when available. Body condition was initially reported qualitatively (Flint et al. 2009a), however this was later reported quantitatively based on a five-point scale (Norton and Wyneken 2015). Earlier clinical records with qualitative BCS, i.e., emaciated, poor,

average and good, were reassigned a score of one, two, three and four, respectively, with five reserved for obese cases. Hydration was evaluated subjectively by assessing the degree to which eyes were sunken, and the dryness of the skin. Buoyancy was assessed by placing the animal in water (when deemed of sufficient strength) and observing for evidence of floating, and difficulty in diving. Appetite was assessed by monitoring whether food offered was eaten and by grading the amount accepted. Although defecation was reported, this was difficult to assess and grade accurately for various reasons (e.g., small volume, liquid consistency, dissipation in water, removal by the filtration system).

Health indicators are dynamic, and may be affected by recent exposure of the sea turtle to a range of factors; such as those associated with transportation, and cold ambient temperatures. Some clinical signs, such as defecation, may also be difficult to assess initially. Given these challenges, the author (using clinical veterinary knowledge), assigned each turtle a separate qualitative grade for each variable. The variables and grades assigned were: mentation (graded as moribund, dull, quiet, normal); appetite (graded as inappetent, reduced and normal); defecation (graded as absent, reduced and normal); and activity levels (graded as moribund, inactive and active, with inactive and active further qualified as weak and strong respectively). The grading scale for haematology and biochemistry results was based on results from the initial assessment. Many turtles had received supportive therapy (e.g., warmth and fluids) prior to the standardised diagnostic investigation, but few had commenced other medical treatment, except for cases rehabilitated in remote areas which were subsequently sent to PZVD for a second opinion. While supportive fluid therapy is expected to cause some changes to blood values, for example haemodilution, rehydration is an essential step and significantly improves recovery rate (Hunt et al. 2019). For consistency, external physical abnormalities and measurements were also conducted and reported under the necropsy external examination (Section 4.3.3), as all necropsies were performed by the author.

Veterinary diagnostics

Blood was collected from the dorsocervical sinus for in-house haematology and biochemistry, and/or sent to the laboratory for analysis. In-house haematology and biochemistry included PCV, TPS and glucose (Chapter 2.2.1). Alternatively, blood was

submitted to Vetpath Laboratory Services (Jandakot Business Park, WA) for a standard zoo reptile blood profile (ZR2), which included testing for PCV, Hb, TWCC and differentials, CK, AST, BUN, uric acid, glucose, sodium, chloride, potassium, total protein, albumin, globulin, calcium, and phosphorus (Chapter 2.2.1). Occasionally, a basic zoo reptile blood profile (ZR1) was performed, which excluded electrolytes. Although serial blood samples were often collected, especially in long-term cases, only blood results from the initial assessment/workup (up to day five) were reported and used for this study's analyses. If sample issues such as haemolysis or clotting precluded the use of the first blood sample, and if the second sample was collected within five days, the second sample was included in the analysis instead.

Blood values were compared to various RIs (Campbell 1996, Kakizoe et al. 2007, Campbell 2014, Kelly et al. 2015, Phillips et al. 2015, Stacy and Innis 2017), including those recently developed for flatback turtles (Chapter 3.3), using the most appropriate species, age-class and life stage RIs (i.e., foraging turtle RIs were used for all stranded turtles, as blood was not collected from the only two pre-ovulatory females [nesting RIs more suitable]). Although this grading has been provided, it does not necessarily represent sea turtle populations in this study, and interpretations should be made with care (Chapter 3.4).

Several diagnostic imaging modalities were used on the live stranded sea turtles, including radiography, ultrasonography and CT. Contrast studies were also undertaken to monitor intestinal motility using BIPS and iohexal contrast, and serial radiographs were taken (Chapter 2.2.1). Radiography is an effective tool for detecting fractures and fishing hooks in sea turtles and was performed following standard protocols (using lateral, dorsoventral and skyline projections). Other imaging modalities such as CT are useful to further evaluate soft tissue structures and abnormalities (Gumpenberger and Henninger 2001, Pease et al. 2017).

Live turtles occasionally also had a parasitology examination performed at Paraquest (Murdoch University Parasitology Diagnostic Service).

Veterinary treatment

A wide range of treatments were provided to the live stranded sea turtles (on a case-by-case basis at the discretion of the attending veterinarian), and the treatments most likely to

impact pathology results were reported; including treatments with antimicrobials and antiparasitic agents.

4.2.3 Dead turtles

Necropsy

For each stranded sea turtle cadaver, a detailed systemic necropsy was performed by a single investigator (the author); and any gross abnormalities were recorded, along with a range of morphometrics and other pertinent data. Most necropsies were performed at Murdoch University (55/75, 73.3%), and these were more detailed than those performed at other locations, including in the field. Specific details of the necropsy procedure are reported in Chapter 2.2.2.

Stranded turtles were categorised according to their manner of death. All turtles found deceased were classed as 'spontaneous' death, and live turtles were categorised either as 'spontaneous death' or 'euthanased'. Stranded turtles were euthanased via an intravenous overdose of barbiturate in a veterinary setting, whereas euthanasia in the field was performed via captive bolt into the cranial cavity.

For necropsy, the cadavers were either kept under refrigerated conditions for up to 72 hours, or were frozen and thawed immediately prior to necropsy. During this study, additional chest freezers were placed at DBCA regional depots to facilitate storage of frozen sea turtle cadavers.

A decomposition score was assigned at the time of necropsy, based on previously established criteria (Flint et al. 2009a), with grading from least autolysed (D1) to most autolysed (D6) (Chapter 2.2.2, Table 2.2); which corresponded to the suitability of the cadaver for diagnostic testing. Usually only fresh D1 and D2 turtles are deemed suitable for detailed histopathological analysis; however, frozen cadavers (D2 or higher) and cadavers up to D4 were also included for histopathology in this study, given the need to freeze cadavers to account for transport delays and extreme environmental conditions in some of the remote areas included in this study. However, D4 cadavers were excluded from most analyses.

Sex was confirmed by gonadal examination at necropsy (as well as through histology). Age-class for the different species was assigned based on categories developed and published in other studies in Australia using CCL (Appendix 17) (Limpus and Limpus 2003, Wyneken et al. 2006, Limpus 2007, 2008c, a, b, 2009, Department of Parks and Wildlife 2013a). In addition to the standard age classes (hatchling, post-hatchling, juvenile, sub-adult, adult), sea turtles were categorised as either immature and mature; with 'mature' representing adults, and 'immature' including all non-adults. The 'immature' category was further differentiated into 'large immature' (sub-adults) and 'small immature' (juveniles and post-hatchlings), using qualitative size evaluations for each species based on the above.

All turtles were weighed, and their body condition was assessed by a single observer (the author), based on previously established qualitative criteria (Flint et al. 2009a) and quantitative criteria (Norton and Wyneken 2015). Body condition was assigned based on a number of criteria at external necropsy examination, including: subjective degree of curvature of the plastron; relative prominence of the bony skull features (including if eyes were sunken); amount of musculature over the neck, shoulders and pelvic region; and muscle tone. Body condition was further assessed through examination of pectoral muscle mass and fat deposits on internal necropsy examination. The qualitative body condition corresponded to a score: 'emaciated' = 0.5-1; 'poor' = 1.5-2; 'average' =2.5-3; 'good' = 3.5-4.5; and 'obese' = 5. Previously established calculations for body condition index (BCI) were also calculated using the formula

$$BCI = \frac{weight\ (kg)}{SCL\ (cm)^3} * 10{,}000 \qquad (1)$$

with associated qualitative scoring: 'poor' = <1.00; 'average' = 1.00-1.10; 'good' = 1.11-1.20, and 'very good' = >1.20 (Bjorndal et al. 2000). While BCI is specifically applicable to green turtles ranging from 30-80 cm, other sea turtle researchers and clinicians use this formula for other species and age/size-classes (Flint et al. 2009b, Norton and Wyneken 2015, Harris et al. 2017).

Amputations were classed according to the extent to which the appendage was affected: graded as 'minor' (1/3 to <1/2 missing), or 'major' (≥1/2 missing). The major amputation grade was also reported when multiple appendages were affected with major or minor

amputations. External damage was only reported as pre-mortem in cases which this could be ascertained with certainty — in live animals or those with corresponding histopathology – and was classified as 'anthropogenic' (which included customary take) or 'natural', including predation (see Section 4.2.4 – Cause of mortality categories, for further details on identifying types of trauma). Many of the sea turtle cadavers presented with minor or old, healed injuries, for example <1/3 flipper missing, or a fully healed minor shell deficit. Such injuries were not reported in this study if they were not considered to have contributed to the morbidity.

Presence of epibiota (i.e., barnacles and algal growth) and grade of epibiota coverage (i.e., density and distribution over the turtle host's skin and shell) were only reported for turtles which had not undergone rehabilitation, as live turtles are placed in a shallow warm freshwater bath to remove epibiota when first admitted into care. In addition, epibiota was inconsistently reported on the stranding form when found initially, and by veterinarians and wildlife rehabilitators at initial examination. Recording of epibiota from frozen cadavers during autopsy also has limited usefulness, as freezing frequently causes epibiota such as barnacles to detach prior to necropsy.

Various organ morphometrics and weights were also recorded during autopsy (data not shown in this chapter). Other morphometrics collected include CCL, CCW, SCL, SCW, HL, HW, *CT*, PT, VT, *SD* (Chapter 2.2.2).

Incomplete cadavers were removed from morphometric analysis in cases where the deficit was expected to impact the measurement; for example, cases with more than half an appendage amputated were excluded from weight analyses, and carapace deficit cases were excluded from CCL reporting and analyses.

Histopathology

Histological samples were collected and placed in 10% NBF. Samples were collected from all major body systems, including: respiratory (lungs); cardiovascular (heart and great vessels); hepatobiliary (liver and gall bladder); digestive (pancreas, oesophagus and GI tract); urogenital (kidneys, gonads, bladder); haematopoietic (spleen and occasionally thymus); lacrimal (salt gland); endocrine (adrenal and thyroid); neurological (brain and sometimes

spinal cord); and occasionally musculoskeletal (muscle and bone). Samples were processed using a standardised approach, and additional staining was performed when required (see Chapter 2.2.3 for detailed methods).

All histopathological slides were examined, and histopathological findings were reported under the supervision of a board accredited veterinary pathologist (Dr N. Stephens, Veterinary Anatomic Pathologist, Murdoch University).

Parasitology

Organ washes were performed to collect digenetic trematodes using 'bucket' parasitology (Prof. D. Blair, James Cook University, personal communication, April 2017). Each organ was separately mixed with chilled saline (8 g/L), and then removed. After the mixture was left for a set period of 20 minutes, the supernatant was decanted and the remaining sediment was examined for trematodes, with a focus on spirorchiids (detailed methods in Chapter 2.2.4). The spirorchiid organ washes that were performed were selected to represent the major body systems, including cardiovascular (heart/great vessels combined), respiratory (lungs), haematopoietic (spleen), hepatobiliary (liver), urogenital (kidneys), lacrimal (salt glands), neurological (brain) and coelomic cavity fluid.

Parasites observed grossly were also collected opportunistically – for example, spirorchiids in thrombotic granulomas in the great vessels. Gastrointestinal, gall bladder and urinary bladder trematodes, as well as GI nematodes were only collected opportunistically on some occasions, as non-pathogenic parasites were not a major focus of this study. It should be noted that while spirorchiids were counted, not every spirorchiid was collected at necropsy; some were left for histopathological analysis, and when representative specimens at a particular site had been saved, collection ceased. However, all parasites were collected when organ washes were performed.

Digenetic trematodes (and occasionally nematodes and metacestodes) were processed for species identification using both morphological and molecular techniques. Parasites were placed in 10% NBF for morphological identification, and into 90% ethanol for molecular work (Chapter 2.2.4). Digital photographs were used to confirm morphological identification of each parasite under magnification by sea turtle trematode expert (Prof. D. Blair, James Cook

University). Occasionally, parasites could be identified on histopathology (Prof. D. Blair, James Cook University). Parasites prepared for molecular analysis were sent to AGRF for Sanger sequencing, and a BLAST analysis was performed in Geneious, followed by a phylogenetic analysis. Species were identified by a molecular diagnostic parasitology expert (Dr T. Miller, DPIRD).

Other testing

Other tests were occasionally conducted in particular instances on the stranded sea turtles; usually for infectious diseases. For example, microbiological culture was performed at DPIRD-DLS (South Perth, WA); this was undertaken infrequently due to the impacts of freezing and post-mortem bacterial overgrowth in frozen and autolysed cadavers, as well as the financial constraints associated with outsourcing diagnostic testing. Electron microscopy was used in a single case to visualise muscle micro-organisms (Chapter 2.2.5). Faecal floatations were performed on samples from live and dead turtles. Various molecular techniques, including next generation sequencing, were also utilised for infectious disease diagnostics. Non-infectious disease testing was performed occasionally; for example, marine debris analysis took place as part of a collaboration with another project, and included sorting, counting, weighing and classifying debris (Dr E. Duncan, University of Exeter, November 2019).

4.2.4 Data analysis

Health status

The health status of each stranded sea turtle was categorised as either 'healthy' or 'unhealthy', to indicate its health condition proximate to stranding, based on ante-mortem and post-mortem findings, with particular consideration to body condition. Turtles categorised as healthy were in good body condition, died suddenly, and had no other pathological findings aside from acute cause of mortality, such as trauma-related predation or vessel strike. Health status was only assigned for turtles which were not in advanced states of autolysis (i.e., D4 turtles excluded), and which had undergone sufficient diagnostic testing. Health status was also assigned to moderately decomposed cadavers, if the

pathology was of such severity, or the body condition so emaciated, that health status could be ascertained; all those for which cause of death was unclear were excluded.

'Cause of stranding' categories

Due to a lack of detail about cause of stranding in the standard stranding form, 'cause of stranding' categories were assigned using data collected at the initial clinical exam or external necropsy exam. Cases were included if the observations could be collected by trained non-veterinary personnel – for example, body condition – and excluded any cases which required specialised veterinary knowledge, skills or equipment.

The 'cause of stranding' category that was assigned to each case included eight categories and represented the preliminary assessment of the primary cause of stranding for that case, and encompassed standard, accepted 'cause of stranding' categories (Manire 2017), including:-

- *Chronic debilitation* – included turtles in poor or emaciated body condition, cachexic, often with heavy epibiota. If alive, turtles were dehydrated, weak and often floating.
- *Trauma* – included turtles with obvious trauma/damage, including amputations. At the stage that this category was assigned, it may not have been clear whether "trauma" occurred pre- or post-mortem.
- *Cold-stunning* – included turtles which stranded due to cold temperatures, often through the misadventure of arriving in cooler waters outside their seasonal range. Cold-stunning can be acute or chronic, leading to poor body condition in chronic cases. Cold-stunned turtles can also present with floating. Storm-blown turtles are only included in this group if also exposed to cold temperatures.
- *Mass mortality event* – included stranded turtles found temporo-spatially together.
- *Buoyancy disorder* – included live turtles found floating and unable to dive. It did not include turtles which were found dead; which naturally float as part of the decomposition process. Turtles were assigned to this category if they did not appear emaciated, or were within their typical geographic range for the cooler months.
- *Entanglement* – included turtles found entangled in marine debris.
- *Fibropapillomatosis* – included turtles with fibropapilloma tumours that could be associated with stranding e.g., periocular fibropapillomas causing vision obstruction.
- *Unknown* – included turtles with no obvious cause of stranding which did not fit clearly into any of the above categories.

'Cause of mortality' categories

Each sea turtle was assigned to one of 12 categories, according to the most likely cause of death, or (if euthanased) likely cause of morbidity leading to euthanasia. Assessment was based on clinical examination, necropsy, histopathology and other veterinary diagnostics. The 12 categories were:

- *Spirorchiidiasis* – assigned to cases with severe systemic spirorchiidiasis or severe neurological spirorchiidiasis. If only the neurological system had a severe rating, neurospirorchiidiasis was assigned (see Spirorchiidiasis grading section below).
- *Trauma* – assigned if pre-mortem trauma was confirmed (for example, haemorrhage identified on histopathology); if there was sufficient circumstantial evidence; or if no other contributory cause was evident in an otherwise healthy animal despite thorough investigation. Specific causes of trauma were further categorised into 'anthropogenic' (including vessel strike or interaction with machinery as indicated by straight incisions or cavitation; and customary or indigenous take/harvest, as indicated by penetrating injury); 'natural' (including predation, indicated by jagged edges and bite marks); or 'unknown' (when the cause of the trauma could not be identified).
- *Endoparasitosis* – assigned to turtles with heavy parasite burdens of the digestive system, including GI cestodiasis (excluding Trypanorhyncha), nematodiasis, trematodiasis, cholecystic trematodiasis and unknown GI endoparasites.
- *Microbiological infectious disease* – assigned to turtles where a pathogenic microbial agent was identified with culture and PCR, with or without associated pathology. Cases that were only positive on PCR were not included in this group, due to the risk of false positives.
- *Pneumonia* – assigned to cases where pneumonia was the most serious observed problem, presenting as discrete granulomas or as diffuse pulmonary changes.
- *Systemic inflammation* – assigned to turtles with evidence of systemic inflammation, indicated by coelomitis, often accompanied with hepatic capsulitis and pleuritis.
- *Gastrointestinal impaction* – assigned to turtles with a severe GI blockage capable of causing mortality.
- *Gastrointestinal foreign body* – assigned only to turtles with GI pathology associated with foreign bodies/marine debris.
- *Osmoregulatory disorder* – assigned to turtles with pathology capable of affecting osmoregulation such as severe salt gland adenopathy.
- *Metabolic disorder* – assigned if metabolic disease was responsible for mortality e.g., gout/pseudogout.
- *Fibropapillomatosis* – assigned to turtles with severe fibropapillomatosis.
- *Unknown* – assigned if a specific cause of mortality could not be determined.

With regards to mortality, the primary body system affected in each turtle was also identified; these included the cardiovascular, respiratory, hepatobiliary, digestive, urogenital, haematopoietic, lacrimal, endocrine, neurological and musculoskeletal systems.

Comparison between 'cause of mortality' category and 'cause of stranding' category

As this study was limited to investigations on deceased stranded sea turtles, for each case the 'cause of mortality' category was compared with the 'cause of stranding' category, in order to ascertain how well the two categories matched each other. In some cases, considerable time may have elapsed between stranding and death (e.g., cases subject to care before death). For these cases, final cause of death may be different from the initial assessment of the cause of stranding; and this was taken into consideration when examining the similarity between the information in the cause of mortality category and cause of stranding category for each case.

Contributory study group

A 'contributory study group' (n=54) was established to represent cases for which it could be ascertained whether disease(s) contributed to the morbidity or mortality; this group included cases both with and without contributory disease. This group excluded all D4 cases (and occasionally D3 cases), as well as all cases for which the primary and/or contributory cause of death was unknown. Also excluded were cases without thorough veterinary diagnostic investigations of all body systems (i.e., incomplete necropsy and histopathology), unless disease(s) could be identified which were likely to have contributed to cause of morbidity or mortality.

Disease duration

Assessment of disease duration was based primarily on body condition, and classified as 'acute', 'subacute' and 'chronic', with overlap between categories. Emaciated turtles or those in poor body condition were generally assigned as 'chronic', and exhibited pathology that was likely chronic in nature. Turtles with acute disease were generally in good or average body condition, and had sustained disease of short duration (< four days), for example traumatic injuries. Subacute duration was defined as a duration between acute and chronic; which generally was days to weeks duration, in contrast to chronic disease, which typically took weeks to months to develop (Stacy et al. 2017a).

Another potential indicator of disease duration, to be used alongside body condition and disease pathology, was ingesta in the stomach. The presence of fresh ingesta may potentially help to differentiate between acute and subacute disease. However, for sea turtles, assessment of ingesta is not a particularly accurate way to assess disease duration, for a number of reasons (Stacy et al. 2017a): turtles may continue to eat when chronically unwell; ingesta may appear fresher than they are, and ingesta may be present due to GI stasis or a blockage. Given the unreliability of stomach ingesta as an indicator of disease duration, this criterion was not used in this study.

As sea turtles are ectothermic animals, immune-suppressive effects of cold temperatures can extend the time it takes to develop chronic disease. Given this, for some analyses this study separated chronic disease from acute and subacute disease, and combined the latter two categories in order to compare them to chronic disease.

Infectious disease

Causes of mortality were also categorised as either 'infectious', 'non-infectious', or 'unknown'. Cases were only categorised as infectious when infectious agents could be detected (i.e., bacteria, fungi, protozoa, parasite) with associated pathology (except for pathogenic bacteria). If cases had signs of microbiological infectious disease but lacked evidence of an infectious agent, they were categorised as unknown; these included cases in the systemic inflammatory disease category discussed above. Fibropapillomatosis cases were an exception, and categorised as infectious without the detection of an infectious agent due to the strong evidence of a viral component in this disease (Boylan et al. 2017a). Diseases which were likely to have contributed to morbidity or mortality were also categorised in the same way as causes of mortality.

It is possible that in some cases, potentially infectious diseases were missed, due to a lack of blood culture and other microbiological testing; however, microbiology was not a focus of this study as frozen cadavers were unsuitable for microbiology and there was limited funding for testing; which had to be outsourced (see above Section 4.2.3 – Other testing for more details).

Spirorchiidiasis study group

A sub-set of sea turtle cadavers (n=44) were selected for spirorchiidiasis testing (spirorchiidiasis study group), comprising sea turtle stranding cases where the cadaver was in suitable condition, and which underwent detailed diagnostic investigation and/or had such severe pathology which explained the cause of mortality. This excluded severely decomposed cases (i.e., D4), as well as most cases with both incomplete necropsy examination and no histopathology. However, cases were still included if a diagnosis could be reached without histopathology by using other veterinary diagnostics, for example microbiology; or if cause of mortality was unknown despite complete necropsy examination, with or without histopathology. Given that the current literature indicates that spirorchiids are not found in oceanic turtles, post-hatchlings were also removed from the spirorchiidiasis study group.

Spirorchiidiasis grading

A spirorchiidiasis grading scale was determined by assessing level of spirorchiid infection and severity of the associated lesions. This scale was applied to all organs/body systems in the spirorchiidiasis study group, using a slightly modified version of previously established guidelines (Flint et al. 2010d) (Table 4.1). It should be noted that in our grading system, if adult spirorchiids were noted, this increased the category rating by one grade (noting severe spirorchiidiasis grading remained a severe grading). Body system spirorchiidiasis grading was predominantly undertaken by histopathology; but where appropriate, gross pathology and parasitology data were also considered. An overall spirorchiidiasis grading was determined for each individual sea turtle by considering all body system grades holistically, alongside the contributory role of spirorchiids in morbidity and mortality (Figure 4.1).

The role of spirorchiidiasis in the cause of mortality, as well as the contributory role of spirorchiidiasis in morbidity and mortality, was also determined based on the overall spirorchiidiasis grade and whether disease was considered contributory or non-contributory to mortality (Figure 4.1).

Although the spirorchiidiasis grading described above was attempted consistently in this study, it is important to acknowledge that assigning significance or contributory status to

spirorchiid-associated pathology is recognised as challenging in the literature for a range of reasons, including the subjectivity of grading and confounding issues such as autolysis. However, all necropsies were performed by one investigator (the author) and all histopathological analyses were performed by the author under the supervision of a single board accredited veterinary pathologist (Dr N. Stephens, Murdoch University), removing potential sources of variation in the results.

Table 4.1 Organ and body system spirorchiidiasis grading.

Grade	Eggs	Pathology	Adults
Severe (3)	Numerous moderate granulomas (≥5 eggs) or large granulomas (≥10 eggs), frequently coalescing. Severe rating includes primary site of egg deposition (typically >100 eggs).	Pathology that includes extensive disruption of architecture such as aneurysm, thrombus, haemorrhage, necrosis, fibrosis, vasculitis and proliferative arteritis.	May be present
Moderate (2)	Several moderate granulomas (5-10 eggs) per x20 field. A moderate rating indicates a secondary site of egg deposition (i.e. egg embolisation).	Some disruption of architecture such as fibrosis and hypertrophy.	May be present
Mild (1)	Zero to several small granulomas (<5 eggs) per x20 field. A mild rating indicates a secondary site of egg deposition (i.e. egg embolisation).	None to minimal pathological changes.	Absent
Absent (0)	No eggs detected	None	Absent

Due to the low numbers of sea turtle stranding cases without spirorchiidiasis (n=3), analytical testing was unable to be performed on this group alone; so the cases without spirorchiidiasis were pooled with cases that were graded as ‘mild’ for some analyses (assigned ‘low’ grade). Likewise, for some analyses, ‘moderate’ and ‘severe’ cases were pooled (assigned ‘high’ grade). Some analyses also compared the ‘contributory’ and ‘non-contributory’ spirorchiidiasis cases.

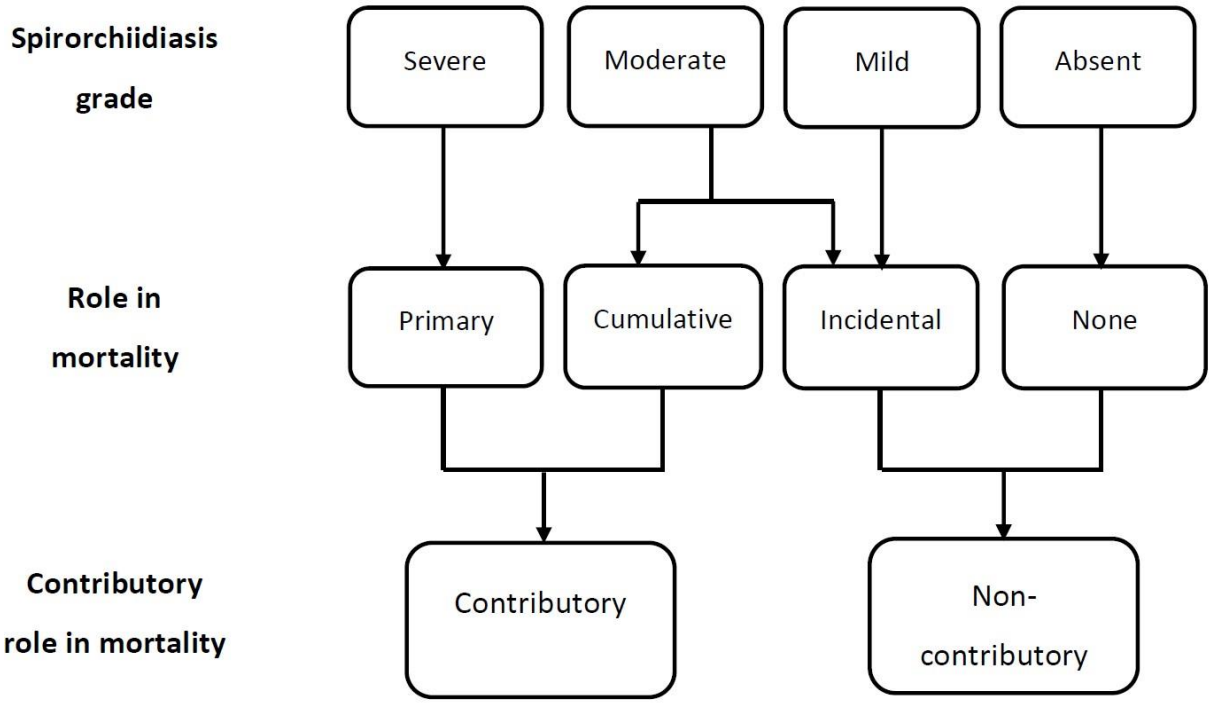


Figure 4.1 Role of spirorchiidiasis in mortality and contributory role in mortality depending on overall spirorchiidiasis grade.

Comparison of ante-mortem and post-mortem results

Where possible, results from ante-mortem examination were compared with post-mortem examination for the same case, to investigate for any similarities or differences, when results from both examinations were available.

Statistical testing

All statistical testing was performed in R (R Core Team 2019). Descriptive statistics were provided for boundary conditions or risk factors including species, age-class, sex, stranding status (live or dead) and cadaver condition, as well as temporal and spatial data such as location and season. Descriptive statistics were also reported for data derived from stranding surveys, rehabilitation notes and veterinary clinical examinations, veterinary diagnostics and treatment, as well as morbidity and mortality investigations, including necropsy, histopathology and parasitology. We also compared how closely the results derived from the different diagnostic techniques used in the morbidity and mortality investigations were associated, including results from necropsy, histopathological tests, and parasitological tests. We further examined how well these results were associated with

ante-mortem results, including stranding surveys and clinical findings. A two-by-two table along with a Pearson's Chi-squared test (used for categorical and frequency data) was used to assess the relationship between risk factors on the morbidity and mortality data (such as body condition, disease duration, affected body systems), and investigate for significant differences between the frequencies of the various groups. Chi-squared analyses were performed on spirorchiidiasis (e.g., incidence and severity of infection, presence of adult spirorchiids, disease contributory to mortality, and body systems affected). If an observed value in any group of the two-by-two table was less than five, Fisher's exact test was used. Given the small sample size of the spirorchiidiasis study group, few statistical analyses were possible and occasionally categories needed to be combined, for example severe and moderate spirorchiidiasis cases were pooled (assigned 'high' grade), and mild and absent groups were also pooled (assigned 'low' grade). Likewise, for body condition, 'emaciated' and 'poor' cases were combined (assigned 'low' score), and 'average' and 'good' cases were combined (reassigned 'high'). Odd ratios were performed if significant differences between the groups were detected, and the odds ratios were presented with associated 95% CIs.

4.3 Results

4.3.1 Study animals

A total of 75 stranded sea turtles underwent necropsy including green (37/75, 49.3%), loggerhead (19/75, 25.3%), flatback (14/75, 18.7%), hawksbill (4/75, 5.3%), and a single olive ridley turtle (1/75, 1.3%). No leatherback turtles were examined. All age-classes (except hatchlings as the study's focus was ocean-dwelling sea turtles which subsequently stranded) were examined including post-hatchlings (27/75, 36.0%), juveniles (36/75, 48.0%), sub-adults (4/75, 5.3%) and adults (8/75, 10.7%) (Table 4.2)**.** This study included female (26/75, 34.7%), male (32/75, 42.7%) and turtles of unknown sex (17/75, 22.6%) (Figure 4.2). Two adult female turtles, including one green and one loggerhead, had flipper tags and nesting histories from Exmouth and Dirk Hartog Island, respectively. All turtles were checked for microchips; however, no microchips were detected.

Table 4.2 Sea turtles investigated by species, age-class and sex.

Species	Green			Loggerhead			Flatback			Hawksbill			Olive ridley			Total
Sex																
Female	16			4			2			4			–			**26**
Male	14			12			5			–			1			**32**
Unknown	7			3			7			–			–			**17**
Age-class	F	M	U	F	M	U	F	M	U	F	M	U	F	M	U	
Post-hatchling	–	–	–	1	10	3	2	4	7	–	–	–	–	–	–	**27**
Juvenile	13	13	6	–	1	–	–	–	–	3	–	–	–	–	–	**36**
Sub-adult	1	1	1	–	–	–	–	–	–	–	–	–	–	1	–	**4**
Adult	2	–	–	3	1	–	–	1	–	1	–	–	–	–	–	**8**
Total	**37**			**19**			**14**			**4**			**1**			

All turtles were found between Esperance in the south and Barred Creek in the North-West (approximately 30 km north of Broome) (Figure 4.3). This spans a distance of 1780 km or 5840 km of coastline. Of the turtles examined, 43/75 (57.3%) animals were found in the north region and 32/75 (42.7%) were found in the south region of the state. Stranded turtles were found in seven of the nine coastal DBCA Districts, with no turtles found in the remote North-East (East Kimberley District), or Mid-West (Mid-West District) parts of the state. Aside from one turtle from Esperance, there were no other turtles found in the South-East (Esperance District).

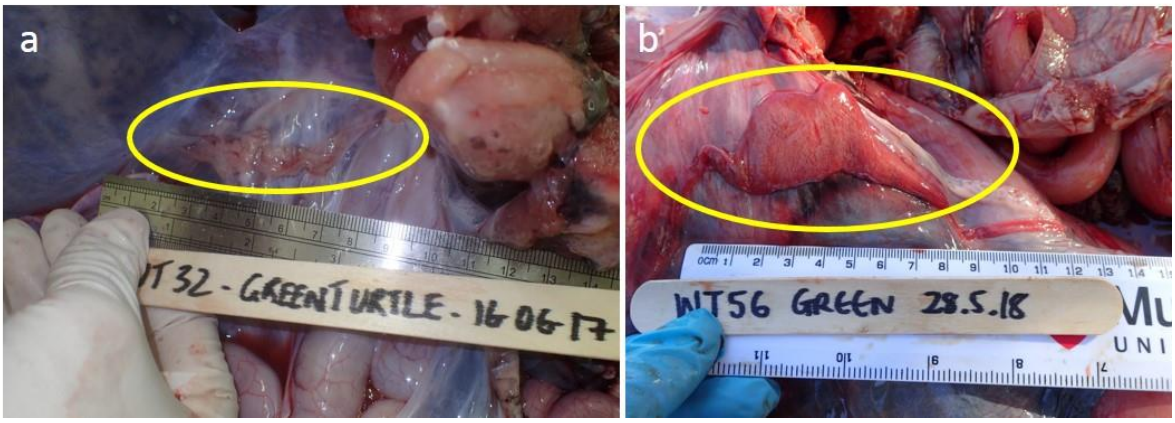


Figure 4.2 Gross anatomical appearance of gonads (circled in yellow) of (a) Juvenile female gonad; and (b) Sub-adult male gonad. As demonstrated in the images, the gonad is much easier to identify in more mature animals.

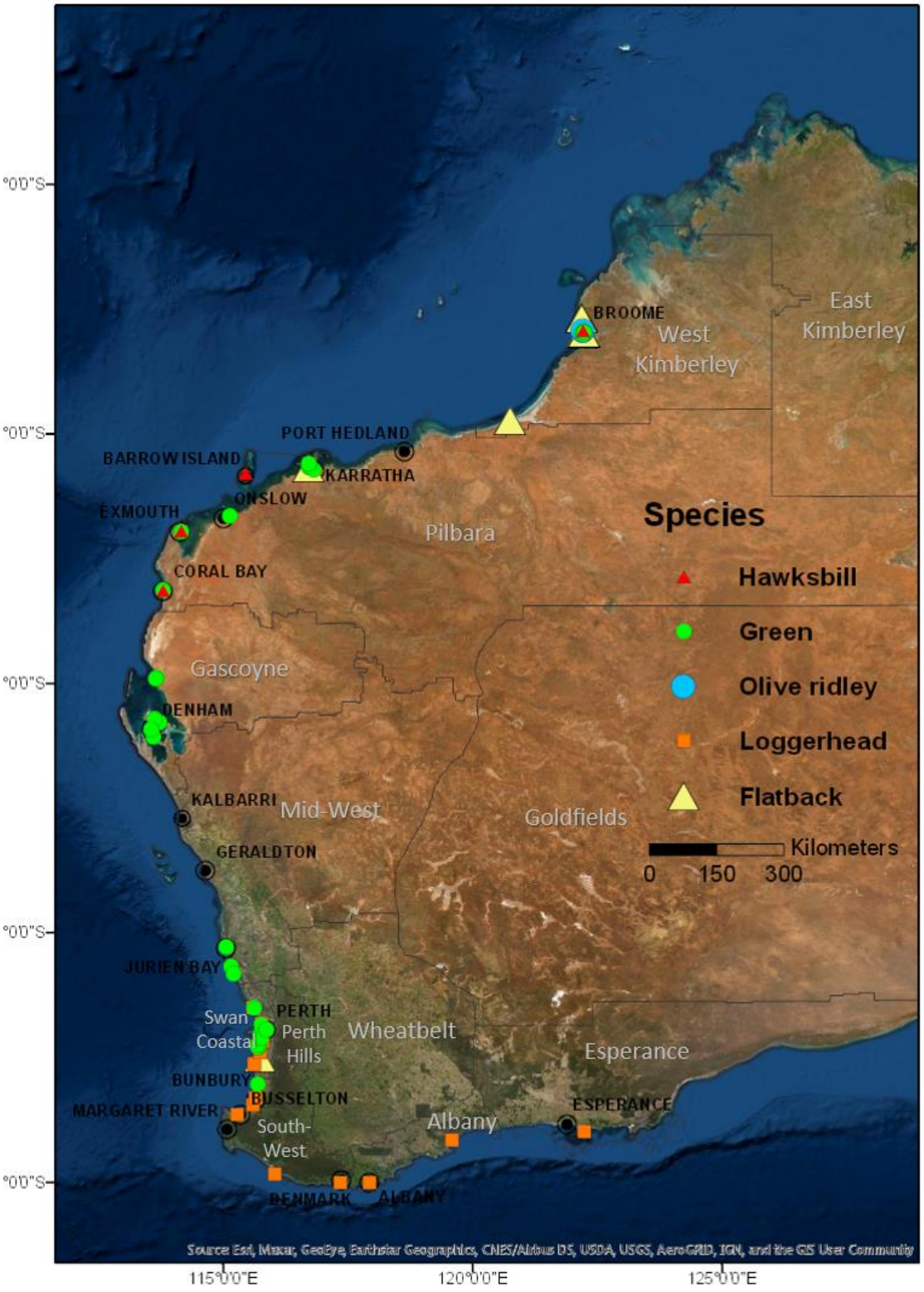


Figure 4.3 Distribution of stranded sea turtles in Western Australia by species (n=75) with Department of Biodiversity, Conservation and Attractions Districts.

Stranded turtles were reported in 2016 (30/75, 40.0%), 2017 (27/75, 36.0%), 2018 (11/75, 14.7%), as well as 2014 (1/75, 1.3%) and 2015 (1/75, 1.3%), and for the remaining turtles the year was unknown but ranged between 2014 to 2018 (5/75, 6.7%). For stranded turtles with known month of stranding (n=68), turtles were found in all months except February (Figure 4.4), with the highest number of strandings reported in August (19/68, 27.9%) and the least in December (1/68, 1.5%). When separated by region (i.e., north and south), the south region (n=32) had one to three stranded turtles retrieved each month (except February) aside from a peak in August (13/32, 40.6%). In the north (n=36), no stranded turtles were collected between December and February and one to three turtles were collected in the other months, except for a peak in March (12/36, 33.3%), with other smaller peaks in August or September (n=6 and n=5 respectively).

When examining seasonal differences of sea turtle stranding frequency by region, most turtles stranded in winter in the south (17/32, 53.1%), whereas stranding was fairly evenly distributed between the dry (19/36, 52.8%) and wet season (17/36, 47.2%) in the north. Turtles in the south were more likely to strand in winter (OR=2.95, 95% CI: 1.1 – 8.31, p=0.035). However, a mass mortality event near Broome in the wet season skewed the distribution (11/17, 64.7%) and if this event was excluded, the peak in the north was also in August (or in the dry season) and regional differences became non-significant ($\chi 2$=0.97, df=1, p=0.325).

At the time of stranding, 45/75 (60.0%) turtles were found alive, 27/75 (36.0%) were found dead and 3/75 (4.0%) were of unknown status. With regards to the location, 35/75 (47.7%) turtles were found on land, 14/75 (18.7%) were found in the water, and the rest were unknown (26/75, 34.7%). All turtles found in the water were floating (11 alive, 3 dead), which is to be expected after a period of decomposition. With regards to who reported and retrieved the turtles, 18/75 (24.0%) were by members of the public, 10/75 (13.3%) were during joint patrol with DBCA and Indigenous ranger groups, 6/75 (8.0%) by DBCA, 2/75 (2.7%) by industry, 1/75 (1.3%) by an Indigenous ranger group, and the rest were unknown (38/75, 50.7%).

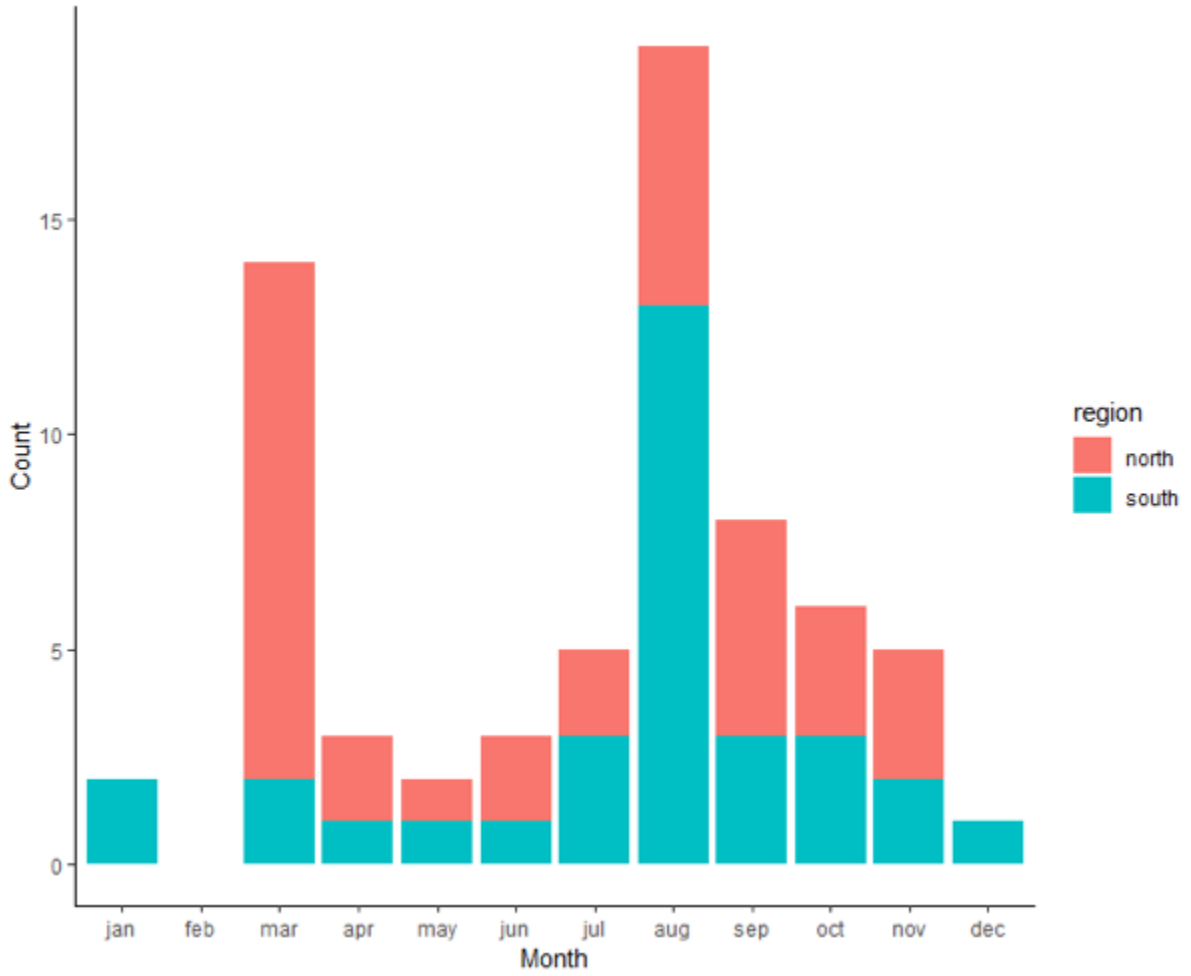


Figure 4.4 Temporal distribution of stranded sea turtles by month and coloured by region from January to December; all years (2016-2018) and species combined (n=68).

Cause of stranding was difficult to determine from the stranding form alone, due to the lack of health-related detail provided; and in some cases no stranding form was completed at all. Stranding-related photos were also rarely available. Only six turtles were reported as poor body condition, and one was reported to be in good condition (7/75, 9.3% reporting). Eight were reported to have a barnacle burden and six were reported with algal growth (total of 9/75, 12.0% of turtles had epibiota reported). For live turtles, eleven turtles were noted to be weak and/or lethargic and/or weakly responsive, five were reported as moribund and one as alert (total of 13/45, 28.9% had clinical signs reported). At the time of stranding, two other turtles were reported to be showing neurological signs such as circling (2/45, 4.4%). Major external abnormalities including injuries or other damage such as amputation of flippers (n=9), as well as trauma of the head (n=4) and carapace (n=3) were also reported on the stranding form (total of 14/75, 18.7% had external abnormalities reported).

Additional data captured on the stranding form revealed eleven turtles were part of a mass mortality event, two turtles were found entangled in fishing-related gear and one turtle was reported with FP. Three turtles appeared to have been hit by a vessel, and three turtles were reported to have injuries consistent with customary take (20/75, 26.7% additional data reported).

4.3.2 Live turtles

Rehabilitation

Rehabilitation was attempted in the majority of live turtles (34/45, 75.6%) at Perth Zoo (16/34, 47.1%), Ocean Park Aquarium (5/34, 14.7%), Chelonia or Native Animal Rescue Broome (5/34, 14.7%), Pilbara Wildlife Carers (3/34, 8.8%), Exmouth Veterinary Clinic (2/34, 5.9%), Albany Wildlife Carers (1/34, 2.9%), Dolphin Discovery Centre (1/34, 2.9%) and Vasse Veterinary Clinic (1/34, 2.9%). Of the 18 turtles which spent the majority of their rehabilitation time in regional locations, 9/18 (50.0%) were sent to Perth Zoo for additional rehabilitation or a second opinion. Average time in rehabilitation was 69.8 days +/- 188.22 (n=32, range: 0-962 days), with most periods of rehabilitation being short (<7 days; 17/32, 53.1%), followed by long (>28 days; 8/32, 25.0%), and mid-term (7-28 days; 7/32, 21.9%) periods (with time in rehabilitation for two turtles unknown). Rehabilitation data were predominantly collected retrospectively.

Veterinary clinical examination

A complete veterinary diagnostic examination was performed on all live stranded sea turtles which presented to Perth Zoo (30/45, 66.7%; six after an extended time in care in regional locations), as well as one turtle assessed by a veterinarian in Exmouth. Twenty-nine of these turtles were weighed and had body condition assessed and a score of one (9/29, 31.0%), two (11/29, 37.9%), three (6/29, 20.7%), 3.5 (1/29, 3.4%) or four (2/29, 6.9%) assigned.

Mentation was described in 30 turtles and was assessed as dull (13/30, 43.3%), quiet (12/30, 40.0%), moribund (4/30, 13.3%) and normal (1/30, 3.3%). For 29 of these turtles, neurological findings were reported, and aside from terminal signs, only six turtles (6/29, 20.7%) in care showed neurological symptoms (plus one additional turtle euthanased in the

field after a vessel collision), which included head tilt (n=2), circling (n=1), flipping over (n=2), ataxia (n=1) and depression (n=3). Levels of activity were reported in 27 turtles and included moribund (4/27, 14.8%), inactive (19/27, 70.4%) and active (4/27, 14.8%), with inactive and active further qualified as weak (n=8) and strong (n=3), respectively. Of the live turtle cases where buoyancy was recorded, seventeen cases exhibited buoyancy disorders during care (17/21, 81.0%; or 23/27, 85.2% if live turtles which had buoyancy reported without veterinary examination were included). Appetite was reported in 17 turtles and described as inappetent (8/17, 47.1%), reduced (8/17, 47.1%), and normal (1/17, 5.9%). Defecation was recorded in 14 turtles and qualified as absent (2/14, 14.3%), reduced (10/14, 71.4%) and normal (2/14, 14.3%). Two turtles reportedly had marine debris in the faeces (2/34, 5.9%). Other reported clinical signs included dehydration (27/34, 79.4%) and hypothermia (4/34, 11.8%).

Veterinary diagnostics

Blood samples

Blood was only collected from live turtles presenting to Perth Zoo. Twenty-two turtles had blood collected, but only 16 were classified as 'on admission'. This included six in-house blood haematology and biochemistry profiles, five laboratory ZR2 profiles, and five samples which had both types of testing undertaken. On admission, a ZR2 blood profile was performed in green (n=4), loggerhead (n=4), hawksbill (n=1) and flatback (n=1) turtles, while in-house on-admission bloods were undertaken in loggerhead (n=6), green (n=4) and hawksbill (n=1) turtles. All loggerhead and flatback turtles sampled were classified as post-hatchlings, all green turtles were classified as juveniles, and the hawksbill turtle was classified as an adult. Six turtles had serial blood samples collected (data not shown). Association of blood results with clinical signs and pathology can be found in Section 4.3.4.

Diagnostic imaging

Various imaging techniques were performed on turtles at Perth Zoo, including radiography (n=22), ultrasonography (n=3) and CT examination (n=1). One other individual had radiographs undertaken at Exmouth Veterinary Clinic.

Contrast studies were undertaken to monitor intestinal motility using BIPS in three turtles, and iohexal contrast in one turtle, but no major blockages or ileus were detected. Six turtles had serial radiographs taken, of which four turtles had a range of contrast studies undertaken.

Of the 23 radiography cases, gas was detected in the GI tract in five cases (5/23, 21.7%), and in the coelomic cavity in three cases (3/23, 13.0%). Radiodense material was detected in the digestive tract of nine cases (9/23, 39.1%). Aside from amputations and fractures, other bony changes were detected in three cases (3/23, 13.0%). An internal tumour was detected in the single fibropapillomatosis case with a pulmonary tumour. Four cases were radiographically normal.

Veterinary treatment

Of the 34 rehabilitated turtles with treatment history (n=29), 14/29 (48.3%) were reported to have received treatment, 13/29 (44.8%) were reported to have received supportive therapy only, and 2/29 (6.9%) were reported not to have received treatment. Eight turtles had treatment prior to initial assessment at Perth Zoo, including six turtles rehabilitated in regional locations prior to arriving at Perth Zoo, and two turtles in care at Perth Zoo.

All turtles which received treatment 14/29 (48.3%) had antimicrobials (12 had 20 mg/kg ceftazidime IM q three days for five treatments [Fortum, Aspen Pharmacare Australia Pty Ltd., St Leonards, NSW] and two had 5 mg/kg enrofloxacin IM SID for five days [Baytril, Bayer Animal Health Pty Ltd., Pymble, NSW]). All but one of these individuals (13/29, 44.8%) also had praziquantel (8 mg/kg, q 14 days for two treatments) and a single case had a nematicide and coccidiostat (1/29, 3.4%). However, only six cases had a complete antimicrobial course and three had a complete praziquantel course, which included two green turtles in long-term care (treatment given two years prior to necropsy).

Of the 45 live animals, 16/45 (35.6%) died spontaneously and 29/45 (64.4%) were euthanased.

4.3.3 Dead turtles

Seventy-five turtles submitted for necropsy examination were frozen (51/75, 68.0%) or fresh (24/75, 32.0%) cadavers. At necropsy, most turtles were in a D2 decomposition state (40/75, 53.3%), followed by D1 (16/75, 21.3%), then D3 (14/75, 18.7%), and finally D4 (5/75, 6.7%). Fresh cadavers ranged between D1-D3, whilst frozen thawed cadavers were D2-D4. All D4 cadavers were part of a mass mortality event.

External examination

Most turtles had body condition assessed at post-mortem examination as poor (30/75, 40.0%), followed by average (22/75, 29.3%), good (13/75, 17.3%), and emaciated (10/75, 13.3%). When compared to the ante-mortem body condition scoring in the veterinary clinical examination mentioned above (n=29), the majority of scores were the same between the two examinations (20/29, 69.0%). For those that were different between the two examinations, the difference was a single grade each time.

Table 4.3 shows the species and age-class, weight, CCL, CCW and BCI, separated by health status including 55/58 (94.8%) unhealthy and 3/58 (5.2%) healthy (see Section 4.3.5 for health status determination results). It should be noted post-hatchling flatback turtles of unknown health status (n=12) were included in Table 4.3 with unhealthy turtles due to the paucity of information in this species.

On external post-mortem examination, 29 turtles had various injuries/damage (29/75, 38.7%). Within this group, 7/29 (24.1%) turtles had grossly observable major deficits (10 dead, 7 live). Flipper amputations were observed in 12/29 (41.4%) turtles (7 dead, 5 live), with multiple flippers impacted in four turtles. Five turtles had minor flipper deficits, while seven turtles had major amputations. When analysing affected flippers within the amputated flipper group, deficits were found in the foreflippers of 10/12 (83.3%); the hindflippers of 5/12 (41.7%); the left side of 9/12 (75.0%); and the right side of 7/12 (58.3%). The left front flipper was affected in 9/12 (75.0%) cases, and the left hindflipper was affected in 2/12 (16.7%) cases. Two live turtles had tail deficits (2/29, 6.9%), while four stranded sea turtle carcasses were found with the rostral head missing (4/29, 13.8%).

Table 4.3 Primary morphometrics of the various sea turtle species (see Appendix 17 for age-class size categories) by health status (only anatomically complete turtles were included for the various measurements, n=70).

	Species	Green			Loggerhead			Flatback		Hawksbill		Olive ridley
Unhealthy turtles	**Age-class**	Juvenile	Sub-adult	Adult	Post-hatching	Juvenile	Adult	Post-hatching*	Adult	Juvenile	Adult	Sub-adult
Weight	n	24	1	1	13	1	2	8	1	3	1	0
	mean / value (n=1)	7.26	52.00	95.60	0.15	38.00	102.80	0.27	65.00	5.73	48.40	
	min	2.30			0.07		82.60	0.11		2.80		
	max	14.30			0.21		123.00	0.61		9.40		
CCL	n	27	1	1	13	1	3	13	1	3	0	1
	mean / value (n=1)	44.04	84.00	103.90	9.79	77.00	102.80	14.02	89.60	41.43		52.50
	min	31.80			7.30		93.60	9.10		35.20		
	max	53.00			12.30		111.00	29.00		49.50		
CCW	n	27	1	1	14	1	3	13	1	2	1	1
	mean / value (n=1)	41.61	81.60	90.70	10.13	73.20	94.00	12.68	76.00	38.40	69.30	57.50
	min	31.40			7.30		90.00	8.50		32.80		
	max	49.60			12.30		99.00	26.00		44.00		
BCI	n	22	1	1	12	1	2	8	1	3	0	0
	mean / value (n=1)	1.00	1.09	1.03	1.98	1.16	1.26	1.04	1.09	0.91		
	min	0.57			1.66		1.19	0.74		0.82		
	max	1.33			2.38		1.34	1.29		0.95		

	Species	Green		
Healthy turtles	**Age-class**	Juvenile	Sub-adult	Adult
Weight	n	1	0	1
	mean / value (n=1)	13.70		104.8
	min			
	max			
CCL	n	2	1	1
	mean / value (n=1)	48	61.2	94.5
	min	45		
	max	51		
CCW	n	2	1	1
	mean / value (n=1)	44	57.1	89.7
	min	42.50		
	max	46.30		
BCI	n	1	0	1
	mean / value (n=1)	1.36		1.55
	min			
	max			

* Unhealthy post-hatchling flatback turtles includes those with unknown health status (n=12) due to the paucity of information in this species.

Other injuries/damage reported within this group included fractures of the flipper (1/29, 3.4%), jaw (1/29, 3.4%), skull (3/29, 10.3%), carapace (4/29, 13.8%) and plastron (3/29, 10.3%), including one skull and three carapace and/or plastron penetrating injuries, as well as skin lacerations (4/29, 13.8%) and ulceration (3/29, 10.3%), deep bony scrapes (1/29, 3.4%) or bony erosions (3/29, 10.3%), and necrosis (1/29, 3.4%). Epibiota was only reported in non-rehabilitated turtles (n=41) and included moderate to heavy algal growth (6/41, 14.6%) and barnacle burden (5/41, 12.2%), predominantly *Chelonibia testudinaria*. As most of the turtles with epibiota reported on the stranding form were subsequently rehabilitated, there was little overlap or consistency in reporting. It should be noted that leeches were not detected during the necroscopic examination throughout this study and were only reported in two live turtles (see Fibropapillomatosis in Section 4.3.4 – Pathological findings), possibly due to the reasons highlighted above (i.e., inconsistent reporting on stranding form and on initial examination, freshwater baths, freezing), however, it is also possible leeches are less common in WA.

4.3.4 Pathological findings

This section is structured according to the stranded turtle 'cause of mortality' categories (Section 4.2.4), with some additional contributory and other diseases included; and is based on necropsy, histopathology, parasitology and other veterinary diagnostics utilised to reach a diagnosis. A complete necropsy was performed in 62/75 (82.7%) turtles. Histopathological analysis was complete in 43/75 (57.3%) cases, incomplete in 18/75 (24.0%) cases, and not performed in 14/75 (18.7%) cases. Organ washes to detect digenetic trematodes, specifically spirorchiids, were performed in 25/44 (56.8%) of the spirorchiidiasis study group (Section 4.2.4), and of these, 20 organ washes were complete (20/25, 80.0%). A total of 183 organ washes were undertaken. Due to the species composition of the spirorchiidiasis study group (green [34/44, 77.3%], hawksbill and loggerhead [4/44, 9.1% for each species], flatback and olive ridley [1/44, 2.3% for each species]), the majority of the organ washes were performed in green turtles (20/25, 80.0%), followed by hawksbill (3/25, 12.0%,) then loggerhead and flatback turtles (1/25, 4.0% for each species). Microbiological studies were performed in a total of 31 individuals (31/75, 41.3%), and included culture +/- PCR in 14 cases (14/31, 45.2%), gram stains in 20 cases (20/31, 64.5%), PAS/PAAS stains in 14 cases (14/31, 45.2%),

and ZN acid-fast stains in five cases (5/31, 16.1%). Faecal floatation tests were performed in a small subset of cases (n=3). Several other cases also had molecular testing performed, which included: PCRs for herpesvirus, papillomavirus, microsporidia; pan-fungal PCR; and next generation sequencing.

Spirorchiidiasis

Histopathological and pathological descriptions

A diagnosis of spirorchiid infection in the 'spirorchiidiasis study group' was usually made by the presence of spirorchiid eggs, typically presenting as small dark raised multi-focal to coalescing masses scattered throughout the viscera, or the presence of the adult spirorchiid, primarily in the main arterial system and usually accompanied with significant pathology such as aneurysm or thrombus (Figure 4.5). Egg masses were primarily seen in the serosal/subserosal surface of the GI tract, with a particular affinity for the duodenum. Aneurysms presented as a smooth dilation of the great vessels and were either cream or black-grey. Thrombi were of various presentation ranging from black to grey and yellow to cream; were sometimes gritty; and in some cases, comprised a mass of adult parasites. Individual parasites were approximately ~2 mm in length and appeared clear and ellipsoid with dark borders. Neurospirorchiidiasis usually presented with dark serpiginous or dendritic, flat to raised lesions; representing egg masses within meningeal vasculature. Occasionally in severe cases, there were meningeal masses which varied in colour and texture; from yellow-cream with granuloma formation, to black-grey and gritty.

Histologically, spirorchiid egg granulomas appeared as groups of yellow to brown-walled eggs, surrounded by a mixed inflammatory reaction; predominantly histiocytic, but often accompanied by lymphocytic-plasmacytic and occasionally granulocytic inflammation, and sometimes with mild fibrosis. Larger, chronic granulomas were typically dominated by multi-nucleated giant cells, and often caused significant disruption of the surrounding cellular architecture (Figure 4.6). The extensive histological damage of the great vessels caused by adult spirorchiids included proliferative arteritis, fibrosis, necrosis, haemorrhage, fibrin deposition and thrombus formation.

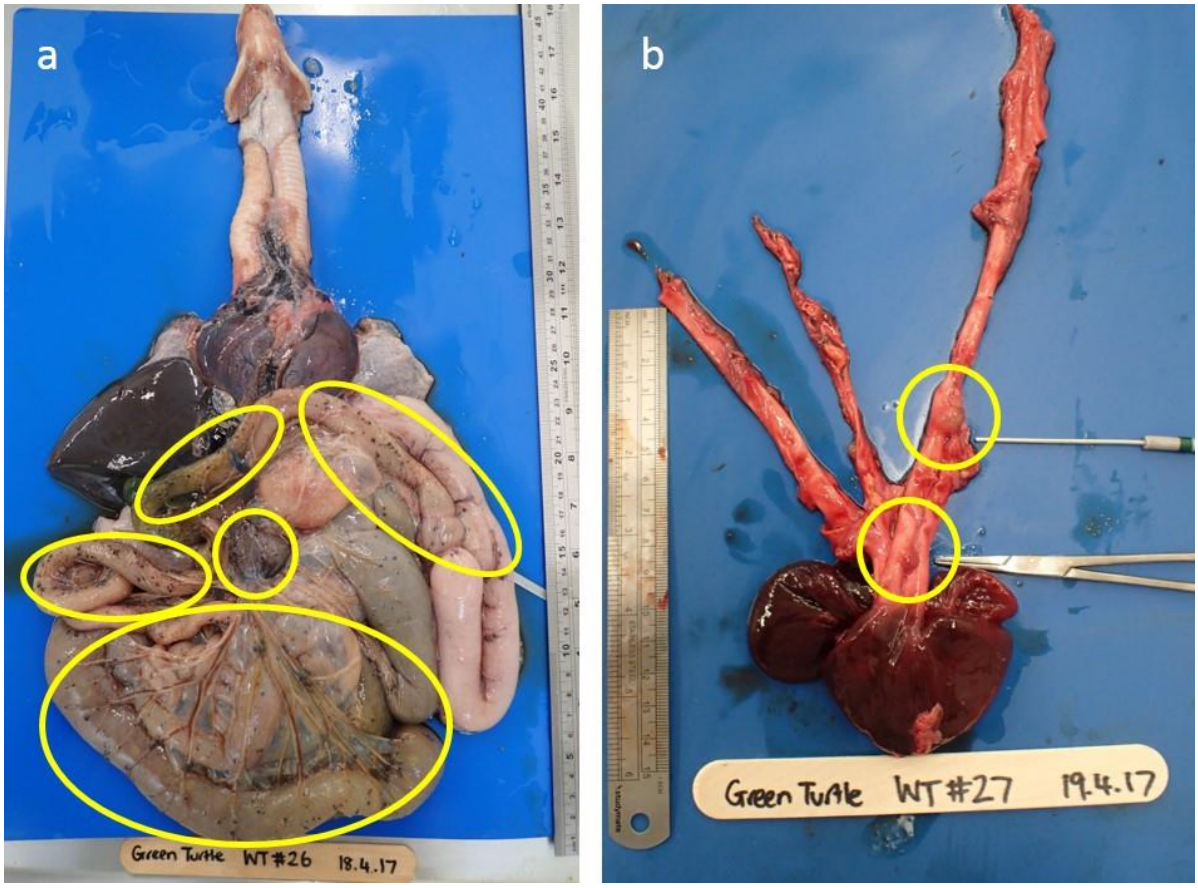


Figure 4.5 Typical gross presentation of (a) Spirorchiid egg granulomas throughout the viscera in systemic spirorchiidiasis; and (b) Multiple aneurysms of the great vessels (circled in yellow).

In neurospirorchiidiasis, spirorchiid egg granulomas were generally located in the meninges, sometimes surrounded by haemosiderophages (possibly from previous haemorrhage). Eggs found within the parenchyma were usually single, isolated and perivascular. Severe cases often had heterophilic granulomatous inflammation and necrosis, and if parasites were present, lesions typically had a stronger reaction (Figure 4.7). Neurospirorchiidiasis in the meninges was usually more severe than in the parenchyma.

Several cases of spirorchiidiasis also appeared to have bacteria and/or fungi associated with spirorchiid eggs (including in the respiratory, digestive and cardiovascular systems). Special stains were usually performed if microorganisms were detected and occasionally microbiology was performed. However, none of the microorganisms detected could be speciated as there was no growth on microbiological culture and special stains are unable to identify microorganisms.

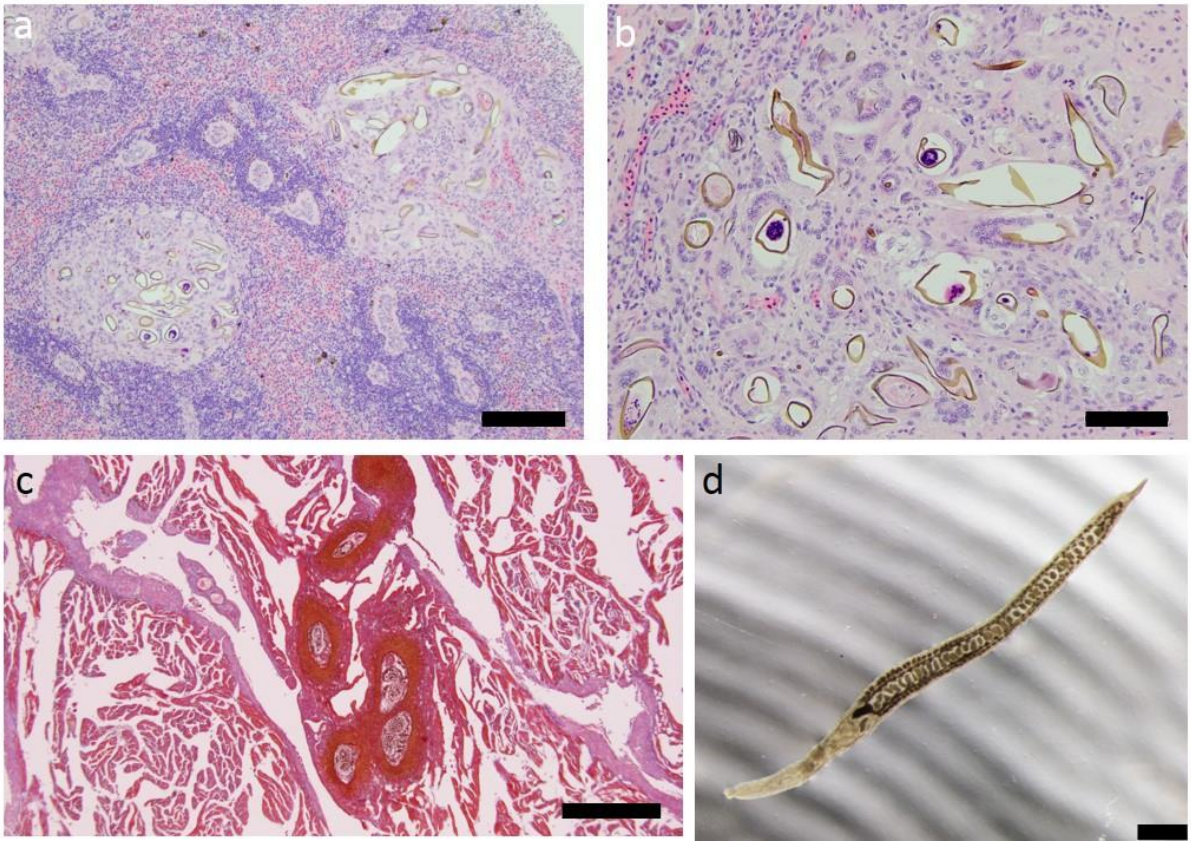


Figure 4.6 (a) Severe granulomatous splenitis with intralesional spirorchiid eggs (H&E, x100, scale bar 200 µm); (b) Severe granulomatous salt gland adenitis with intralesional spirorchiid eggs (predominant cells are multi-nucleated giant cells) (H&E, x200, scale bar 100 µm); (c) Photomicrograph of the heart showing spirorchiid (*Neospirorchis* sp.) migration tract and fibrosis, along with granulomatous reaction to parasites (MSB, x40, scale bar 500 µm); and (d) An example of a spirorchiid found in a liver wash (scale bar 500 µm), noting this was not included in the study as it was outside the study period but was found in a flatback from WA (WT82).

Parasitological investigations

Thirty-six adult spirorchiids were collected from 19/183 (10.4%) positive washes in 10/25 (40.0%) sea turtle stranding cases (including eight green and two hawksbill turtles). Cases in the spirorchiidiasis study group that did not have spirorchiidiasis (n=3) each had the full range of spirorchiid detection methodologies performed (necropsy, histopathology and organ washes), and all detection methods were negative. With these negative cases removed (three cases including 21 organ washes), this resulted in a slightly higher success rate of spirorchiid detection in organ washes of 19/162 (11.7%) and 10/22 (45.5%) cases than with the negative cases included. Within the spirorchiid positive organ wash group, parasites were found most frequently in the haematopoietic system (5/22, 22.7%), followed

by the respiratory system (4/21, 19.0%), the cardiovascular system (4/22, 18.2%), the hepatobiliary system (4/22, 18.2%), the renal system (1/19, 5.3%) and finally the neurological system (1/22, 4.5%). It should be noted that multiple species of spirorchiid were found in three of the combined heart and great vessel organ washes (Table 4.4). No spirorchiids were detected in the salt gland or coelomic cavity organ washes.

Due to the unique life cycle of the flatback turtle (no oceanic phase), a single post-hatchling flatback turtle also had a complete set of organ washes (except the brain), which returned negative results. However, this case was excluded from the study group when all post-hatchlings were removed.

A further 25 spirorchiids were collected on necropsy from the cardiovascular system in five green turtles, one of which was also positive for spirorchiids on the corresponding cardiovascular organ wash.

Eggs were collected either at necropsy or during organ washes from six other turtles which did not have spirorchiids collected (from the brain in four cases, the salt gland in one case, and from both the spleen and brain in one case).

Two faecal tests, performed on an adult hawksbill (WT46) and a juvenile green turtle (WT26), were positive for spirorchiid eggs, possibly *Hapalotrema* or *Learedius* spp.

Spirorchiid species identification

Of the 26 representative spirorchiid samples from the total of 51 collected (26/51, 51.0%), 13 were identified morphologically, seven were identified through molecular techniques, and six were identified using both methods. Seven spirorchiid egg samples were processed: five were identified using molecular techniques; and two were identified by morphology (Table 4.4).

Sixteen spirorchiid specimens were identified to species, nine to genus level, and one to family level (i.e., Spirorchiidae). When the spirorchiid identification findings obtained using molecular techniques were compared with the findings using morphology, the two techniques were found to have produced the same results in 100% of cases. Identifications of spirorchiid included three different species, and four different genera.

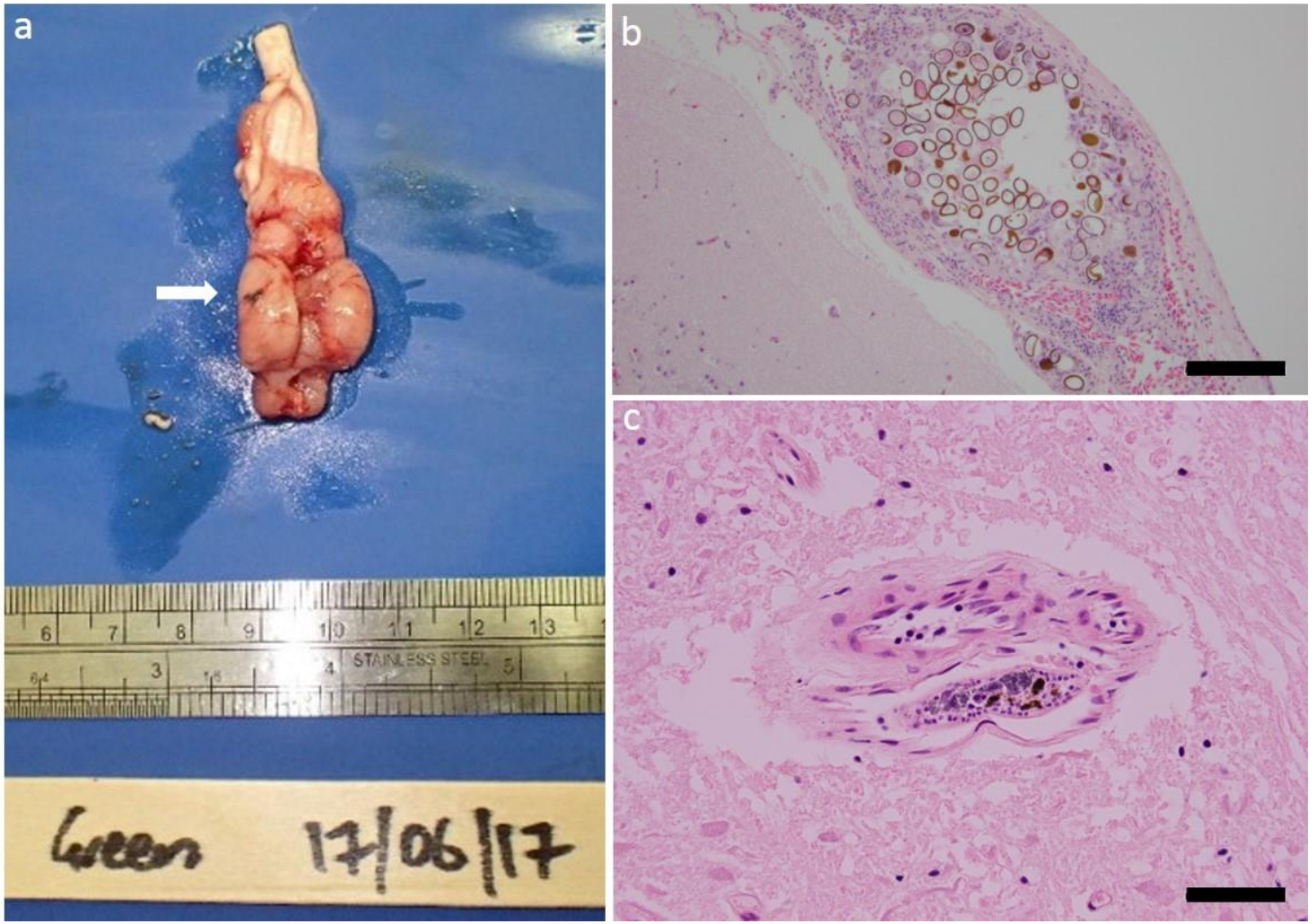


Figure 4.7 (a) Gross neurospirorchiidiasis in juvenile green turtle (WT33), with arrow indicating spirorchiid granuloma in meningeal vessels; (b) Histological presentation of neurospirorchiidiasis of same turtle as in (a) showing spirorchiid-egg associated granulomatous meningitis (H&E, x100, scale bar 200 µm); and (c) Photomicrograph of perivascular spirorchiid adult in flatback turtle (WT28) brain (H&E, x400, scale bar 50 µm).

Six turtles were observed to have the same spirorchiid species or genus present in multiple organs, and four turtles were found to have multiple spirorchiid species/genera in multiple organs; of which two cases were found to have different spirorchiid species/genera simultaneously inhabiting the same body system (in both cases, this was the cardiovascular system) (Table 4.4). The most commonly observed spirorchiid was *Hapalotrema mehrai* (10/26, 38.5%) which was also the most widespread with respect to different body systems; this species was observed in four body systems in the same individual sea turtle. If speciated eggs are included in the analysis, *Neospirorchis* sp. was also found in four body systems. The turtle with the highest diversity of spirorchiid species was observed with *H. mehrai, Learedius learedi*, and *Neospirorchis* spp. All eggs were identified as *Neospirorchis* spp., with the exception of eggs observed in the salt gland; which were identified as either *Hapalotrema* or *Learedius* spp.

As speciated eggs and parasites were not collected from the same sea turtle stranding case, this study could not examine whether eggs and parasites in a stranded sea turtle case were the same species.

When considering regional differences in spirorchiid presence in sea turtle stranding cases from the north and south, all species and genera of spirorchiids identified in this study were found in sea turtles that originated in the north, while for cases from the south, only *H. mehrai, H. mistroides, Hapalotrema* and *Neospirorchis* spp. were found (no *Carettacola* or *Learedius* spp. were observed).

A total of 61 endoparasites were collected during necropsy and organ washes. As expected, the majority were collected from the cardiovascular system (42/61, 68.9%), followed by the respiratory system (7/61, 11.5%), the haematopoietic and hepatobiliary systems (5/61, 8.2% for each system), and finally the renal and neurological systems (1/61, 1.6% for each system). As collection of adult spirorchiids during necropsy was not performed in a systematic way, spirorchiid quantities were only an estimate; and cannot be assumed to represent frequencies.

Table 4.4 Spirorchiid species detected in sea turtles, including collection and identification methods.

ID	Species	Age-class	Sex	Location	Health status	Body system	n	Collection method	Identification method	Species
Adults										
WT7	Hawksbill	Juvenile	♀	Coral Bay	Unhealthy	Hepatobiliary	1	Organ wash	Molecular	*Learedius learedi*
WT9	Green	Adult	♀	Monkey Mia	Unhealthy	Cardiovascular	10	Necropsy	Morphological	*Hapalotrema* sp.
						Renal	1	Organ wash	Molecular	*Carettacola hawaiiensis*[a]
						Haematopoietic	1	Organ wash	Morphological	*Carettacola* sp.
WT26	Green	Juvenile	♂	Exmouth	Unhealthy	Haematopoietic	1	Organ wash	Morphological	Unknown spirorchiid spp. (Spirorchiidae)
WT27	Green	Juvenile	♀	Wedge Island	Unhealthy	Haematopoietic	1	Organ wash	Morphological	*Hapalotrema* sp.
WT31	Green	Juvenile	♂	Shark Bay	Unhealthy	Cardiovascular	1	Organ wash	Molecular	*Hapalotrema mehrai*
						Respiratory	3	Organ wash	Morphological & Molecular	*Hapalotrema mehrai*
WT34	Green	Sub-adult	♀	Point Peron	Unknown	Cardiovascular	5	Organ wash	Morphological & Molecular	*Hapalotrema mehrai*
						Hepatobiliary	1	Organ wash	Morphological	*Hapalotrema mehrai*
						Respiratory	1	Organ wash	Morphological	*Hapalotrema mehrai*
						Haematopoietic	1	Organ wash	Morphological	*Hapalotrema mehrai*
						Cardiovascular	(1)	Organ wash	Morphological	*Cricocephalus* sp. (pronocephalid)[b]
WT43	Green	Juvenile	♀	Karratha	Unhealthy	Cardiovascular	>1	Histopathology	Morphological	*Neospirorchis* sp.
WT48	Green	Juvenile	♀	Coogee	Unhealthy (subacute)	Cardiovascular	3	Necropsy & organ wash	Morphological & Molecular	*Hapalotrema mistroides*
						Cardiovascular	7	Organ wash	Morphological & Molecular	*Hapalotrema mehrai*
						Hepatobiliary	1	Organ wash	Molecular	*Hapalotrema mehrai*
						Respiratory	2	Organ wash	Morphological & Molecular	*Hapalotrema mistroides*
WT52	Green	Juvenile	♀	Shark Bay	Unhealthy	Cardiovascular	5	Organ wash	Morphological & Molecular	*Hapalotrema mistroides*
WT54	Green	Juvenile	♀	Broome	Unhealthy	Haematopoietic	1	Organ wash	Molecular	*Learedius learedi*
WT57	Green	Juvenile	♂	Coral Bay	Unhealthy	Neurological	1	Organ wash	Molecular	*Learedius learedi*
						Cardiovascular	1	Organ wash	Morphological	*Hapalotrema mehrai*
						Cardiovascular	1	Organ wash	Molecular	*Neospirorchis* sp.

ID	Species	Age-class	Sex	Location	Health status	Body system	n	Collection method	Identification method	Species
						Hepatobiliary	2	Organ wash	Morphological	*Learedius* sp.
WT71	Green	Juvenile	♂	Bunbury	Unhealthy	Cardiovascular	1	Organ wash	Morphological	*Hapalotrema* sp.
WT74	Hawksbill	Juvenile	♀	Broome	Unhealthy	Respiratory	1	Organ wash	Morphological	*Hapalotrema mehrai*
WT75	Green	Juvenile	♀	Karratha	Unhealthy	Cardiovascular	8	Organ wash	Morphological	*Hapalotrema* sp.
Eggs										
WT5	Green	Juvenile	♂	Exmouth	Unhealthy	Neurological		Necropsy	Molecular	*Neospirorchis* sp.
						Haematopoietic		Organ wash	Molecular	*Neospirorchis* sp.
WT18	Green	Juvenile	♂	Exmouth	Unhealthy	Neurological		Necropsy	Molecular	*Neospirorchis* sp.
WT28	Flatback	Adult	♂	Karratha	Unhealthy (subacute)	Neurological		Necropsy	Morphological	*Neospirorchis* sp.
WT32	Green	Juvenile	♀	Monkey Mia	Unhealthy	Lacrimal		Organ wash	Morphological	Type I egg (*Hapalotrema* or *Learedius* spp.)
WT50	Loggerhead	Adult	♀	Point Peron	Unhealthy (subacute)	Neurological		Necropsy & organ wash	Molecular	*Neospirorchis* sp.
WT56	Green	Sub-adult	♂	Carnarvon	Unhealthy (subacute)	Neurological		Necropsy	Molecular	*Neospirorchis* sp.

[a] BLAST species matches 99.9% for pairwise and identical sites with *Carettacola hawaiiensis*, a species previously only thought to be found in Hawaii, later discovered in Taiwan and Queensland, Australia.

[b] Locating a pronocephalid in the heart is an unusual finding and likely occurred post-mortem.

In one case, a pronocephalid (GI trematode) *Cricocephalus* sp. was observed from a heart wash (from a dead turtle from which other spirorchiids were also collected); the presence of this organism in the heart is unusual, and its occurrence in this location was likely post-mortem. In another case, a spirorchiid was identified as *Carettacola hawaiiensis* by BLAST (species match 99.9% for both pairwise identification and identical sites), previously only thought to be found in Hawaii but was later discovered in Taiwan and Queensland, Australia (Chen et al. 2012, Chapman et al. 2016b, Chapman et al. 2017).

Comparison of findings by spirorchiid detection method

It is useful to consider the consistency of results of spirorchiid positive turtle cases by spirorchiid detection methods, including histopathology and necropsy. With respect to the relative proportions of positive spirorchiidiasis cases identified, histopathology detected all positive spirorchiidiasis cases (40/40, 100%) for which histopathological examination was undertaken, whereas necropsy detected 32/41 (78.0%) cases, including a single case where histopathology had not been undertaken. With respect to the relative proportions of cases positive for spirorchiid eggs, histopathology detected spirorchiid eggs in all positive cases, and necropsy detected eggs in 30/41 (73.2%) cases. With respect to the relative proportions of cases positive for adult spirorchiids, histopathology detected adult spirorchiids in 20/40 (50.0%) cases, and necropsy detected adults in only 5/41 (12.2%) cases. With respect to examining the relative proportions of only cases positive for adult spirorchiids on histopathology or necropsy, necropsy detected adult spirorchiids (in the same body system) in only 4/20 (20.0%) of the positive histology cases, while histopathology detected adult spirorchiids (in the same body system) in 4/5 (80.0%) of the positive necropsy cases. Differences between detection of adult spirorchiids grossly and histologically could potentially be related to all specimens having been collected for parasitological analysis, prior to histological analyses being undertaken.

Further comparison of different spirorchiid detection methods were performed by comparing the consistency of results from organ washes with the results of histopathology and necropsy. All but one of the stranded turtle cases with positive organ washes were positive for spirorchiidiasis on histopathology and necropsy (9/10, 90% for each group). With respect to the relative proportions of cases with positive organ washes, histopathology only

detected adult parasites in the same body system as 4/19 (21.1%) positive organ washes (from four individuals), only one of which was also positive on necropsy (1/19, 5.3%). Conversely, organ washes detected adult spirorchiids in the same body system as histopathology in 4/11 (36.4%) cases (one positive histopathology body system was removed from the analysis due to a missing organ wash), and in the same body system as necropsy cases in 1/2 (50.0%) cases.

When comparing results of the different methodologies for cases with spirorchiid positive organ washes (n=22), washes detected adult spirorchiids in 10/22 (45.5%) positive cases, histopathology detected 7/22 (31.8%), and necropsy detected 2/22 (9.1%). Further analysis involved including only cases for which all the different adult spirorchiid detection methodologies were available for each body system (i.e., histopathology and organ washes), this resulted in removal of: the endocrine system and the digestive system results from the analysis due to missing organ washes from these body systems; coelomic cavity results due to missing histopathology; and results from other cases due to missing organ washes (n=6, including the removal of one positive case for adult spirorchiids on histology). Following the removal of these cases, we found although detection rates were low, organ washes were more successful at detecting adult spirorchiids (19/148, 12.8%), as compared to histopathology (11/148, 7.4%) and necropsy (2/148, 1.4%).

Combined pathology, histopathology and parasitology results

Using the combined methodologies, evidence of spirorchiidiasis was found in 41/44 (93.2%) of the spirorchiidiasis study group, with absence reported in only three cases (3/44, 6.8%). This figure of 93.2% is used to represent the incidence of spirorchiidiasis in this study. Using all methodologies combined, adult spirorchiids were detected in 27/44 (61.4%) of the spirorchiidiasis study group, which equates to 27/41 (65.9%) when negative cases were excluded.

The most common presentation of spirorchiidiasis in this study was the presence of spirorchiid egg granulomas (41/41, 100%,) followed by spirorchiid-associated pathology in the great vessels (25/41, 61.0%). This spirorchiid-related great vessel pathology was only reported in green turtles and the single flatback turtle, and turtles presented with thrombi

+/- aneurysms in the great vessels which were either singular (10/25, 40.0%) or multiple (15/25, 60.0%). Thrombi were found predominantly in the descending aorta (11/25, 44.0%; side not recorded), followed by the left aorta (7/25, 28.0%), then the right aorta (3/25, 12.0%), however, location was not recorded in 10 cases (10/25, 40.0%).

When examining body systems of positive spirorchiidiasis cases, using results from the combined methodologies, the body systems most frequently affected by spirorchiidiasis included the haematopoietic system (39/41, 95.1%), the respiratory system (38/40, 95.0%), and the digestive system (38/40, 95.0%). When examining the body systems of positive spirorchiidiasis cases for the presence of adult spirorchiids, using results from the combined methodologies, most adult spirorchiids were derived from the cardiovascular system (15/41, 36.6%), followed by the neurological system (10/36, 27.8%), the haematopoietic system (6/41, 14.6%), the respiratory system (5/40, 12.5%), the hepatobiliary system (4/39, 10.3%), the urogenital system (2/38, 5.3%) and finally the digestive system (including mesenteric vessels) (2/40, 5.0%) (Table 4.5). Further, each individual examined had multi-organ spirorchiidiasis.

When grading the intensity of the spirorchiid infection of the positive cases within the spirorchiidiasis study group, the body systems with severe as the predominant rating (i.e., '3') were the cardiovascular system (27/41, 65.9%), followed by the neurological system (19/36, 52.8%), then the haematopoietic system (19/41, 46.3%) and digestive system (18/40, 45.0%). Body systems which had mild spirorchiidiasis as the most common spirorchiid infection intensity grading were the respiratory system (20/40, 50.0%), the hepatobiliary system (19/39, 48.7%), the urogenital system (22/38, 57.9%), the lacrimal system (16/36, 44.4%), and the endocrine system (12/35, 34.3%), whereas the predominant spirorchiid infection intensity grading for musculoskeletal was absent (14/25, 56.0%). When the spirorchiid intensity grading for each body system was averaged over all cases, the cardiovascular system was highest, followed in descending order by the haematopoietic system, digestive system and neurological system. All stranded sea turtles had an average spirorchiidiasis grading of 0-3 (Table 4.5).

When combining the results of the spirorchiidiasis grading for all body systems, the overall spirorchiid burden was determined to be severe in 24/44 (54.5%) cases, cumulative (or

moderate) in 9/44 (20.5%) cases, incidental in 8/44 (18.2%) cases, and absent in 3/44 (6.8%) cases. In all severe spirorchiidiasis cases, spirorchiidiasis was identified as the primary cause of mortality. Five of the cumulative cases had moderate spirorchiidiasis which was considered to be contributory to cause of death. The other four cumulative cases also had moderate spirorchiidiasis which was considered to be pathological but non-contributory to the individual animal's death. As expected, spirorchiidiasis detected in the incidental (or mild) spirorchiidiasis cases was also found to be non-contributory to death.

Spirorchiidiasis by sea turtle species

Spirorchiid infection was found in all five species of sea turtle examined, including 31/34 (91.2%) greens, all loggerhead and hawksbill (100%, n=4 for each), and the single flatback and olive ridley turtles (100%, n=1 for each). The three turtles without spirorchiidiasis were all green turtles (3/34, 8.8%). When all methodologies were combined, adult spirorchiids were found in 19/44 (43.2%) green, 3/44 (6.8%) loggerhead and 3/44 (6.8%) hawksbill turtles, as well as in the single flatback and olive ridley turtles (1/44, 2.3% for each). When analysing body systems affected in green turtles, adult spirorchiids were found in the cardiovascular system (14/34, 41.2%), the haematopoietic system (6/34, 17.6%), the neurological system (5/34, 14.7%), the respiratory system (4/34, 11.8%), the hepatobiliary system (3/34, 8.8%), and the urogenital and digestive system (2/34, 5.9% for each).

Adult spirorchiids were found in the cardiovascular system, the respiratory system, and the liver of three different hawksbill turtles (3/44, 6.8% for each). Adult spirorchiids were also found in the neurological systems of three loggerhead turtles (3/44, 6.8%) and the single flatback and olive ridley turtles (100%, n=1 for each).

Generally, spirorchiid species were identified through parasitology, but in some cases, species could be identified on histopathology. With respect to species of spirorchiid found in the different sea turtle species, all species and genera of spirorchiid were found in green turtles, whereas only *Neospirorchis* spp. were found in flatback and loggerhead turtles (as eggs), and *H. mehrai* and *L. learedi* were found in hawksbill turtles (Table 4.4).

Table 4.5 Body systems affected by spirorchiidiasis and average spirorchiidiasis grading, separated by species of sea turtle.

All species Body system	Respiratory	Cardiovascular	Hepatobiliary	Digestive	Urogenital	Haematopoietic	Lacrimal	Endocrine	Neurological	Musculoskeletal
Total tested	43	44	42	43	41	44	39	38	39	28
Spirorchiidiasis positive	38	36	29	38	34	39	30	27	28	11
% positive	88.4%	81.8%	69.0%	88.4%	82.9%	88.6%	76.9%	71.1%	71.8%	39.3%
Average spirorchiidiasis grade	1.52	2.10	0.92	1.77	1.06	1.88	1.19	1.20	1.74	0.50
SD spirorchiidiasis grade	1.07	1.24	0.90	1.19	0.82	1.16	1.04	1.04	1.33	0.86
Green										
Total tested	33	34	32	33	32	34	29	29	29	21
Spirorchiidiasis positive	29	29	23	30	26	30	21	20	19	10
% positive	87.9%	85.3%	71.9%	90.9%	81.3%	88.2%	72.4%	69.0%	65.5%	47.6%
Average	1.56	2.38	0.97	1.91	1.09	2.04	1.22	1.22	1.52	0.59
SD	1.07	1.13	0.94	1.19	0.87	1.18	1.15	1.12	1.35	0.93
Hawksbill										
Total tested	4	4	4	4	4	4	4	4	4	3
Spirorchiidiasis positive	3	2	3	2	3	3	3	2	3	2
% positive	75%	50%	75%	50%	75%	75%	75%	50%	75%	66.7%
Average	1.13	1.00	1.00	1.25	0.63	1.50	1.13	1.00	2.00	0.00
SD	1.31	1.41	0.82	1.50	0.48	1.29	1.03	1.15	1.41	0.00
Loggerhead										
Total tested	4	4	4	4	4	4	4	4	4	2
Spirorchiidiasis positive	4	3	2	4	4	4	4	4	4	1
% positive	100%	75%	50%	100%	100%	100%	100%	100%	100%	50%
Average	1.88	0.88	0.75	1.50	1.00	1.38	0.88	1.25	2.50	0.50
SD	1.31	0.85	0.96	1.22	0.71	0.75	0.25	0.50	1.00	0.71

Body system	Respiratory	Cardiovascular	Hepatobiliary	Digestive	Urogenital	Haematopoietic	Lacrimal	Endocrine	Neurological	Musculoskeletal
Flatback										
Total tested	1	1	1	1	1	1	1	1	1	1
Spirorchiidiasis positive	1	1	1	1	1	1	1	1	1	0
% positive	100%	100%	100%	100%	100%	100%	100%	100%	100%	0%
Average/Value	1	3	0.5	1	2	0.5	2	1	3	0
Olive ridley										
Total tested	1	1	1	1	0	1	1	0	1	0
Spirorchiidiasis positive	1	1	0	1		1	1		1	
% positive	100%	100%	0%	100%		100%	100%		100%	
Average/Value	1	1	0	1		1	1		3	

When comparing the severity of spirorchiidiasis in the various body systems for the different sea turtle species, the highest intensity spirorchiidiasis grading in green turtles was the cardiovascular system, whereas in all other sea turtle species, although sample sizes were small, the highest intensity spirorchiidiasis grading was the neurological system, equal only to the cardiovascular system in the flatback turtle (Table 4.5).

Spirorchiidiasis by age-class/size and sex

When considering age-class, of the positive spirorchiidiasis cases within the spirorchiidiasis study group, 34/41 (82.9%) were immature and 7/41 (17.1%) were mature animals. Adult spirorchiids were found in 23/44 (52.2%) immature and 4/44 (9.1%) mature turtles. The smallest turtle with spirorchiidiasis (WT71) ('incidental' spirorchiidiasis grade) was a green turtle found in Bunbury (-33.0379, 115.6789). This turtle measured 31.8 cm CCL and weighed 2.30 kg, and was classified as emaciated. The largest turtle without spirorchiidiasis was a green turtle (WT30) found at Penguin Island (-32.3049, 115.6913), measured 45.0 cm CCL and weighed 11.5 kg (missing >1/2 foreflipper, estimated at 12 kg with a complete flipper). As expected, there was no evidence of spirorchiidiasis in any post-hatchlings (n=22 with D4 cases excluded).

When considering sex, of the positive spirorchiidiasis cases within the spirorchiidiasis study group, 21/41 (51.2%) turtles were female, 17/41 (41.5%) were male, and 3/41 (7.3%) were of unknown sex.

Spirorchiidiasis by region and season

When considering spirorchiidiasis by region and season, significantly more spirorchiidiasis cases were detected in the north region than the south region (p=0.034); whereas season did not have an effect on spirorchiidiasis (p=1). Of the cases positive for spirorchiidiasis within the spirorchiidiasis study group, 29/41 (70.7%) were found in the north region and 12/41 (29.3%) in the south region. Across both regions, 26/41 (63.4%) spirorchiidiasis positive cases were found in the cool season, and 8/41 (19.5%) positive cases were found in the warm season; the season was not recorded for the remaining cases (7/41, 17.1%) and was listed as 'unknown'. When separated by region, in the north region 18/29 (62.1%) spirorchiidiasis positive cases were found in the cool season, 4/29 (13.8%) were found in the

warm season, and 7/29 (24.1%) were listed as 'unknown' season. In the south region, 8/12 (66.7%) were found in the cool season, and 4/12 (33.3%) were found in the warm season. The negative cases were all found in the south region, two in the cool season and one in the warm season.

When examining severity of spirorchiid infection with region and season, there were significantly more 'high' grade spirorchiid infections in the north region (p=0.001) when compared to the south region; with 'high' grade spirorchiidiasis 28.0 times more likely in the north region than the south region (OR=24.50, 95% CI: 3.64-495.19, p=0.005). Season did not have an effect on the severity of spirorchiidiasis (p=0.327).

Risk factor associations

When considering the various risk factors (age-class, sex, region, season) that were analysed for association with spirorchiidiasis variables, including presence and severity of spirorchiidiasis, the only risk factor found to be associated with incidence and intensity of spirorchiid infection was region.

Within the spirorchiidiasis study group, spirorchiidiasis was the primary cause of death for different sea turtle species including 18/34 (52.9%) green, 3/4 (75.0%) loggerhead, 1/4 (25.0%) hawksbill and 100% for both the flatback and olive ridley turtles (n=1 for each). When examining region for all sea turtles combined in the spirorchiidiasis study group, the overall average spirorchiidiasis score was 2.66 for the north region (n=29), and 1.6 for the south region (n=15). On further examination of spirorchiidiasis as the primary cause of death by region, 20/29 (69.0%) cases were found in the north region (including 15 immature green turtles, one mature green turtle, one mature hawksbill turtle, one mature flatback turtle and one immature olive ridley turtle); and 4/15 (26.7%) cases were found in the south region (including two immature green turtles and two mature loggerhead turtles). When comparing incidence of contributory spirorchiidiasis and non-contributory spirorchiidiasis by region, statistically significant differences were found by region ($\chi 2=10.75$, df=1, p=0.002), with contributory spirorchiidiasis 4.8 times more likely in the north region than in the south region (OR=9.6, CI: 95% 2.42-44.87, p=0.002).

With respect to correlations between body systems and spirorchiidiasis incidence and intensity, each body system showed statistically significant correlations for incidence with each body system, except for the musculoskeletal system and the hepatobiliary system (Table 4.6 and Table 4.7). However, when examining severity categories of spirorchiidiasis combined to two levels (i.e., 'low' and 'high' grade spirorchiidiasis) in each body system, severity of spirorchiidiasis in the respiratory system, the cardiovascular system, the digestive system, the haematopoietic system and the lacrimal system were each statistically correlated. The neurological system and the endocrine system were also significantly correlated with these body systems, except neither were correlated with the cardiovascular system. When considering the effect of spirorchiidiasis in the various body systems on body condition combined to two levels (i.e., assigned 'low' and 'high' score), the haematopoietic system showed a statistically significant correlation with low BCS ($\chi 2=4.13$, df=1, p=0.042).

Neurospirorchiidiasis

The stranded sea turtles in the spirorchiidiasis study group were examined for the presence of neurospirorchiidiasis. Of the positive spirorchiidiasis cases, 28/36 (77.8%) were positive for neurological spirorchiidiasis, and 10/36 (27.8%) had adult parasites; but when examined for the presence of spirorchiids in the neurological system, 10/28 (35.7%) had adult parasites (Table 4.5). In the neurological system organ washes, only one parasite was found, *L. learedi*, as well as *Neospirorchis* sp. eggs.

In the positive spirorchiidiasis group, neurospirorchiidiasis was detected in green (19/26, 73.1%), loggerhead (4/4, 100%), hawksbill (3/4, 75%), flatback and olive ridley turtles (100%, n=1 for each). Of these neurospirorchiidiasis cases, 22/28 (78.6%) were found in the north region and 6/28 (21.4%) in the south region. With regards to season, 18/28 (64.3%) neurospirorchiidiasis cases were found in the cool season and 5/28 (17.9%) were found in each of the warm and unknown seasons. When examining age-class and sex of the neurospirorchiidiasis cases, 22/28 (78.6%) were immature and 6/28 (21.4%) were mature, while 16/28 (57.1%) were female and 12/28 (42.9%) were male.

Table 4.6 Statistical significance of the correlation for the presence of spirorchiidiasis between each body system (all p-values are derived from Fisher's exact test except those indicated with an asterix (*) which are from Chi-squared test).

	Respiratory	Cardiovascular	Hepatobiliary	Digestive	Urogenital	Haematopoietic	Lacrimal	Endocrine	Neurological
Respiratory									
Cardiovascular	**<0.001**								
Hepatobiliary	**0.028**	**0.006**							
Digestive	**<0.001**	**<0.001**	**0.028**						
Urogenital	**<0.001**	**<0.001**	**0.016**	**0.002**					
Haematopoietic	**0.008**	**0.002**	**0.002**	**<0.001**	**0.028**				
Lacrimal	**<0.001**	**0.009**	**0.003**	**0.007**	**<0.001**	**0.007**			
Endocrine	**<0.001**	**<0.001**	0.052*	**0.001**	**0.001**	**<0.001**	**0.001**		
Neurological	**<0.001**	**0.001**	0.092*	**0.017**	**<0.001**	**0.017**	**0.001**	**<0.001**	
Musculoskeletal	0.125	0.191	0.099	0.125	0.355	0.132	**0.023**	0.090	0.190

Table 4.7 Significance of the correlation for the severity of spirorchiidiasis (separated into 'high' grade and 'low' grade) between each body system (asterix (*) indicates p-values are derived from Chi-squared test, with the remaining results derived from Fisher's exact test).

	Respiratory	Cardiovascular	Hepatobiliary	Digestive	Urogenital	Haematopoietic	Lacrimal	Endocrine	Neurological
Respiratory									
Cardiovascular	**0.019**								
Hepatobiliary	**0.030**	0.451							
Digestive	**0.028**	**0.003**	0.790*						
Urogenital	**0.037**	0.064	0.441	0.491					
Haematopoietic	**0.001**	**<0.001**	1.000	**<0.001**	0.154				
Lacrimal	**0.002**	**0.006**	0.696	**0.019**	**0.007**	**0.005**			
Endocrine	**0.006***	0.770	1.000	**<0.001**	0.225*	**<0.001**	**<0.001**		
Neurological	**0.008**	0.303*	1.000	**0.007**	0.151	**0.042***	**<0.001**	**<0.001**	
Musculoskeletal	0.087	0.530	1.000	0.087	1.000	0.533	0.635	0.030	0.225

When examining neurospirorchiidiasis as the cause of mortality (5/75, 6.7%), there was one case in each species where the disease was found to be the cause of mortality. Three of these cases were adult sea turtles and two were immature (specifically large), and four were found in the north region and a single case in the south region. Two were found in the cool season, two were found in the warm season, and one case was from the unknown season. Of these five cases with severe neurological spirorchiidiasis, four had adult parasites detected in the brain.

Associations between ante-mortem results and pathological and parasitological findings

Stranded sea turtles in the spirorchiidiasis study group were examined for associations between ante-mortem clinical findings and post-mortem pathological and parasitological findings. Turtles with spirorchiidiasis showed non-specific signs including cachexia, quiet mentation, lethargy, weakness and buoyancy disorders. The only turtle diagnosed with neurospirorchiidiasis which had a neurological exam (WT46), was neurologically normal until peri-mortem seizures occurred. For the other six turtles with severe neurological spirorchiidiasis, which had been assigned a different mortality category (i.e., systemic spirorchiidiasis, fibropapillomatosis and hepatobiliary trematodiasis), 3/6 (50.0%) displayed abnormal neurological signs and the other 3/6 (50.0%) were normal. Signs ranged from severe (head tilt, circling, head tick) to mild (quiet mentation). When examining the 24 turtles with mild/absent neurological spirorchiidiasis (24/44, 54.5%), 20 were neurologically normal (20/24, 83.3%), and four (4/24, 16.7%) were abnormal.

When examining stranded turtles for associations between the results from diagnostic imaging and post-mortem pathological and parasitological results, one green turtle (WT5) was reported to have a spirorchiid gas pattern on radiography. The adult hawksbill (WT46) had signs of spirorchiidiasis detected on CT, as evidenced by chronic pulmonary arteritis, however a CT of the brain was negative, and IV contrast media would likely be required for confirmation of neurospirorchiidiasis, which was unable to be administered at the time.

Treatment associations with pathological and parasitological findings

Stranded sea turtles were examined for associations between treatment regimens and pathological and parasitological results. Praziquantel was provided to 8/20 (40.0%) live turtles in the spirorchiidiasis study group. Of these individuals, three had adult spirorchiids (3/8, 37.5%). All three sea turtles were treated in regional locations and had incomplete medical records, including incomplete anthelmintic treatment regimes. For the untreated group (12/20, 60.0%), seven had adult spirorchiids (7/12, 58.3%). The effect of treatment on the presence of adult spirorchiids was not statistically significant for the different treatment groups ($\chi 2=0.83$, df=1, p=0.361). Furthermore, turtles without spirorchiids had no treatment.

Trauma

Of the stranded sea turtle cases with significant external abnormalities, 20/29 (69.0%) of these cases were deduced to have occurred pre-mortem (or 20/75, 26.7% of the total cases). Flipper amputations were found in 7/20 (35.0%) cases (four major and three minor amputations), including the left foreflipper (n=5), the right foreflipper and hindflipper (n=2 for each) and the left hindflipper (n=1). Other major trauma included fractures of the carapace (4/20, 20.0%), plastron (3/20, 15.0%), skull (3/20, 15.0%) and flipper (2/20, 10.0%) (an additional case with a fractured scapula was detected on internal necropsy, WT1), as well as tail deficits (2/20, 10.0%). Other significant external abnormalities include major skin ulceration (3/20, 15.0%), lacerations (4/20, 20.0%), necrosis (1/20, 5.0%), and bony erosions (5/20, 25.0%).

Trauma in stranded sea turtles was assessed to determine likely cause; including both natural and anthropogenic causes. Flipper amputations were attributed to natural causes (4/7, 57.1%) and anthropogenic causes (3/7, 42.9%), including vessel strike, customary take and entanglement. For five turtles, it was not possible to determine whether flipper amputations had occurred pre- or post-mortem. This resulted in pre-mortem flipper amputations in 7/70 (10.0%) of the stranded turtle cases. Fractures of the skull (n=3) were attributed to anthropogenic causes (vessel strike, customary

take), and flipper fractures (n=2) were either natural causes or unknown. Carapace fractures (n=4) were either from customary take or unknown reasons, and plastron fractures (n=3) were either customary take, natural causes, or unknown. Lacerations (n=4) were attributed to natural causes or anthropogenic causes (the latter being either vessel strike or customary take). Severe skin and shell ulceration (+/- necrosis) (n=7, some cases with both skin and shell disease) was due to anthropogenic causes (entanglement), natural trauma or unknown causes, and in some cases potentially related to cold-related immunosuppression, and even chronic debilitation; with one turtle (WT54) presenting with deep plastron ulceration due to muscle wastage and the exposure of the acromium processes of the shoulder girdle.

Fibropapillomatosis

Descriptions of the stranded sea turtles with FP are provided, including both ante-mortem results and pathological and parasitological findings. The two sea turtle cases with FP were both kept in captivity in a regional rehabilitation centre for more than two years. The first case (WT33) was found entangled in a fishing trap. The second case (WT32) was found with mild periocular FP four months after the first case arrived, and placed in the same tank as its conspecific on arrival. One month later, the first case (WT33) started to show signs of FP. It should also be noted that leeches were reported to be present on each of the turtles during their time in care. Both sea turtles were sent to Perth Zoo for a second opinion regarding the FP, and due to the deterioration of the second case (WT32). Following diagnostic investigations on initial presentation at Perth Zoo, both individuals were euthanased the day of arrival, due to the severity of disease and severity of tumours, which were deemed inoperable. Fibropapillomatosis was diagnosed in both cases by presence of cutaneous or visceral tumours, including fibromas, papillomas and fibropapillomas; and graded using a scoring system developed by Work and Balazs (1999) and Rossi et al. (2009). The turtles had an average of 53.5 +/-16.26 external tumours (n=2, range: 42-65 external tumours), and an average size at the widest point of the tumour of 3.5 cm +/- 3.16 (n=107, range: 1-28 cm), which were extensively distributed; covering the skin of the

neck, shoulders, flippers, tail, cloaca, inguinal and periocular areas, as well as the carapace and plastron scute seams and conjunctiva (Figure 4.8). The turtle in worse condition (WT32) also had ocular tumours. Tumours displayed varied morphology, colour and texture. Tumours were cream, pink or grey; ranged from single solitary tumours to multi-lobulated to multifocal to coalescing; varied from smooth to verrucous to papillary projections; and were sessile to pedunculated and regular to irregular shape. Necrosis, ulceration and haemorrhage of the tumours was also present. Internally, both turtles also had pulmonary lesions (see below for histological diagnoses). Using the Work and Balazs (1999) scoring system, both turtles had a grading of three; however, using the new scoring system (Rossi et al. 2016), the turtle in worse body condition (WT32) likely had a score of four, due to the ocular tumours hindering vision.

Figure 4.8 Images showing the extent of the fibropapillomatosis in two green turtles (WT32 and WT33). (a) Green turtle (WT32) ante-mortem with fibropapillomatosis affecting the cervical, axillary, inguinal, cloacal and periocular regions, as well as the eyes (photo credit Ocean Park Aquarium). (b) Green turtle (WT33) at necropsy showing large, ulcerated tumours on the neck and shoulders of the cranial half of the body (left foreflipper missing).

Histologically, the fibropapilloma tumours showed mixed orthokeratosis, parakeratosis, acanthosis, acantholysis, vacuolar degeneration and ballooning, with bacteria in accumulated keratin/mucin within dermal-epidermal clefts. Some tumours also showed erosive, mixed heterophilic-granulocytic inflammation with fibrino-

necrotic material covering coccoid bacteria. Spirorchiid ova were a common finding in the cutaneous fibropapilloma tumours. The ocular tumours were classed as fibromas, due the mucosa missing from the sample and histological section; and contained moderate spirorchiid egg granulomas. Pulmonary and great vessel intimal fibromas were present in one turtle (WT32). The pulmonary fibroma was immature and consisted of undifferentiated and disorganised stroma with mild spirorchiid egg granulomas, while the intimal tumour contained aggregates of mixed inflammation, including lymphocytic-heterophilic-histiocytic and multi-nucleated giant cells admixed with cells with brown intracellular deposits (haemosiderophages/ melanomacrophages). The other turtle (WT33) had pulmonary lesions consistent with mild multi-focal chronic granulomatous interstitial pneumonia, where some granulomas had eosinophilic cores with opportunistic fungi.

Aside from the severe FP, both turtles had high BCS (3.5-4.5), despite the second case (WT32) becoming inappetent. However, this body condition is to be expected, considering they were in captivity and fed regularly.

Although herpesvirus and papillomavirus PCRs, as well as next generation sequencing, were performed on the tumours, all results were negative.

Streptococcus iniae

In March 2016, prior to the commencement of this study, a mass mortality event occurred in north-west WA, approximately 30 km north of Broome. This mass mortality event involved numerous species of fishes, sea turtles and sea snakes; and subsequent disease investigations found evidence that the bacterium *S. iniae* was associated with the mortalities (see Chapter five). During this mortality event, 12 post-hatchling flatback turtles were found dead. Of these post-hatchlings, 11 were necropsied (and included in this study), and two of these cases had histopathology undertaken. Microbiological culture and molecular diagnostics were performed in nine cases, on samples from liver, kidney, spleen, heart and brain. Three cases were positive for *S. iniae* on culture and PCR (all tissues types except the brain were positive on

culture, while liver and heart were positive on PCR), whereas one case had a positive liver PCR only (see Chapter five for details). Due to autolysis, no associated pathology was detected on histopathology; and despite the additional use of special stains, only indeterminant bacteria were detected.

Pneumonia and other pulmonary pathology

Despite the high incidence of pulmonary pathology (42/62, 67.7%) among stranded sea turtles which had the lungs examined thoroughly (i.e., excluding cases lacking histopathology without gross pulmonary pathology), only cases where the pathology was deemed to be a contributory factor in mortality were classified as pneumonia. Pneumonia presented as either discrete, firm cream nodules, sometimes with a caseous tan core, or as diffuse pulmonary changes; for example, emphysema, oedema and haemorrhage. Histologically, granulomas were primarily granulocytic, and showed a combination of necrosis and fibrosis with fibrin deposition. Sometimes infectious agents (e.g., bacterial, fungal, parasitic) were present, either as primary or opportunistic pathogens. At other times the cause of the pulmonary pathology was no longer evident. Nodules were located equally frequently on the left, right and bilaterally (n=3 for each). Diffuse changes were accompanied by hyperplasia, hypertrophy, fibrosis, granulocytic or histiocytic inflammation, and in some cases, associated with intrafaveolar nematode eggs/larvae. At other times nematode eggs were present in airways with no associated pathology. Nematode eggs detected in the lung were identified on histopathology as possibly *Angiostoma carettae* (Prof. D. Blair, James Cook University, personal communication, 5 December 2019).

Of the 14 stranded sea turtle cases which had special histological stains performed on the pulmonary tissue (14/60, 23.3%), mycotic pneumonia was detected in 8/14 (57.1%) cases and bacterial pneumonia was detected in 2/14 (14.2%) cases with special histological stains (Figure 4.9). Only four pneumonia cases had ZN acid-fast stain performed to exclude mycobacteria, and all four were negative. Nematode eggs were also detected in six cases, and classified as verminous pneumonia in the four cases with associated pathology. In terms of mixed infections, a mixed bacterial and

verminous pneumonia (spirorchiid eggs) was detected in one individual, while mixed fungal and verminous pneumonia (spirorchiid eggs) were detected in four individuals. In one mycotic pneumonia cases, bacteria (small tetrad cocci, possibly *Sarcina* spp.) were also detected in the lungs, however, it was not considered to be a contributory cause of morbidity.

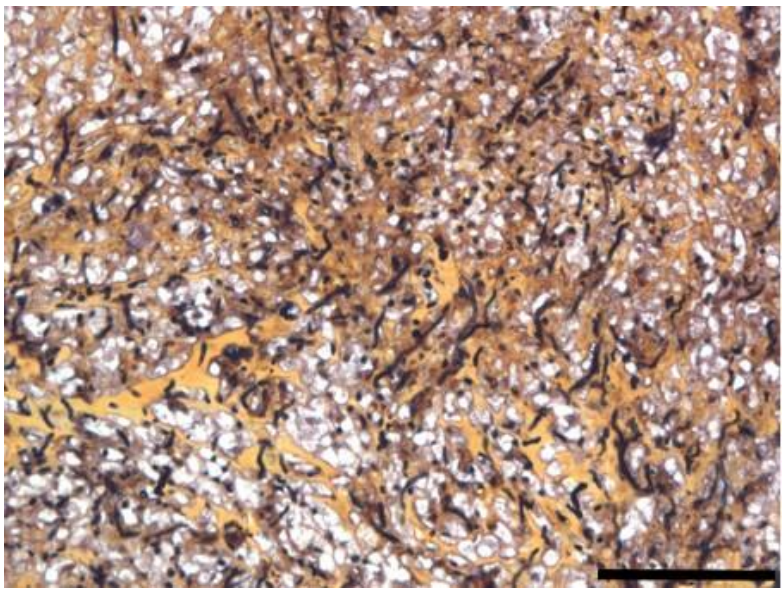


Figure 4.9 Special stain showing fungal hyphae stained black in pulmonary fungal nodule from green turtle (WT13) (PAAS, x200, scale bar 100 µm).

While drowning is difficult to confirm, one turtle attacked by a predator, had bilateral pulmonary emphysema and oedema, and also had evidence of gritty material through the parenchyma, and likely drowned secondarily to a predator attack. Five other cases had other evidence of drowning (e.g., gritty material in lung, foamy material in trachea) however, these were not as compelling as the former case, and probably occurred post-mortem.

Endoparasitosis

When examining stranded sea turtles for internal parasites, on histopathology, post-hatchling loggerhead turtles were found to have mixed, heavy GI parasite burdens. Cestodes, including the tetraphyllidean plerocercoids, were associated with multi-focal erosions and mixed inflammation in the intestinal tract (Figure 4.10). Nematodes,

such as migrating *Anisakis* sp. larvae, were identified within the intestinal wall (Prof. D. Blair, James Cook University, personal communication, 5 December 2019), with associated pathology including fibrosis and disruption of architecture.

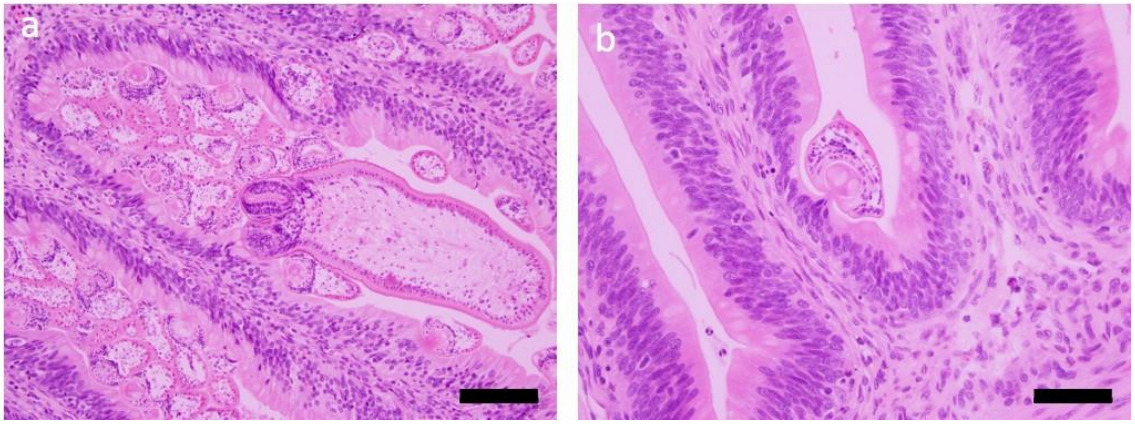


Figure 4.10 (H&E) (a) Post-hatchling loggerhead (WT70) with severe cestodiasis showing tetraphyllidean plerocercoids (larvae) in the intestinal crypts (x200, scale bar 100 µm), with possible other cestode species present; and (b) Post-hatchling loggerhead (WT69) with tetraphyllid attached to the mucosal wall (x400, scale bar 50 µm).

Adult nematodes were identifiable grossly in the intestinal tract of a juvenile (WT58) and in the GI tract of adult loggerhead turtle (WT49), identified as *Sulcacaris sulcata* in the juvenile and *Kathlania leptura* in the adult (J. Mitchell, DPIRD, personal communication, 20 June 2018). The juvenile loggerhead turtle showed moderate to severe multi-focal to coalescing ulcerative and heterophilic fibrinous entero-colitis, associated with the nematodes invading the intestinal wall. In the adult loggerhead turtle, despite the extreme nematode burden and hyperaemic mucosa evident on necropsy, enteritis was only mild on histology.

Two juvenile green turtles also had evidence of nematodiasis due to larval *Anisakis* sp. (Prof. D. Blair, James Cook University, personal communication, 28 March 2019). One turtle (WT30) had a solitary, transmural, fibro-necrotic intestinal granuloma, and the other turtle (WT40) had no grossly observable pathology, but on histopathology there was a mixed inflammatory reaction and moderate burden of larval *Anisakis* sp. Despite the associated pathology, neither incidence of nematodiasis was considered contributory to mortality.

Four turtles, including juvenile loggerhead (WT58), hawksbill (WT74) and green (WT75) turtles, as well as an adult loggerhead (WT49), had evidence of cholecystitis and/or hepatitis due to gall bladder fluke infestation. These cases each had either a necrotic, ulcerative, exudative cystitis with mural fibrosis, or periductular hepatitis with necrosis and fibrosis (or had severe autolysis). One of these cases (WT49) also had green joint fluid and intimal surfaces stained green (hyperbiliverdinaemia) (Figure 4.11). This can occur for various reasons, including hepatic disease, haemolytic anaemia or emaciation (Anderson et al. 2017); this particular case was most likely related to hepatic fasciculitis and periductular inflammation associated with trematodiasis (despite the heavy nematode burden mentioned above).

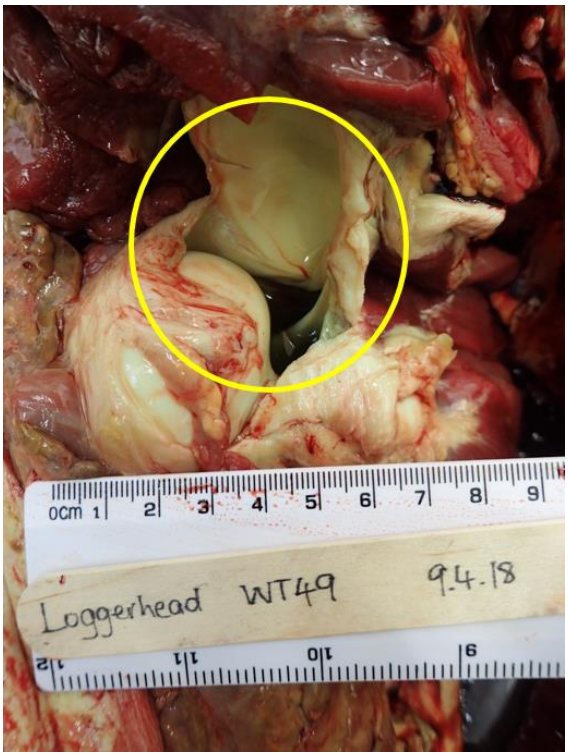


Figure 4.11 Hyperbiliverdinaemia showing green-tinged fluid in the joint space.

When considering the types of parasite species in the hepatobiliary systems of stranded sea turtles, gall bladder flukes were collected in one turtle (WT74) and were identified as *Calycodes* sp.; these are usually found in the GI tract. Flukes reported in the hepatobiliary system of other turtles included *Rhytidodoides* sp. in the gall bladder (WT27) and *Enodiotrema carettae* in the large bile ducts (WT58).

As normal inhabitants of the GI tract, trematodes were observed regularly during sea turtle necropsies; and eggs were occasionally detected on faecal floatation. Species detected include microscaphid (*Deuterobaris* sp.), paramphistome (*Schizamphistomum* sp.), echinostomatid (*Rhytidodes* and *Calycodes* spp.), plagiorchiid (*Orchidasma amphiorchis*) and a number of pronocephalids (*Cricocephalus*, *Pleurogonius*, *Diaschistorchis* and *Adenogaster* spp.) (Prof. D. Blair, James Cook University, personal communication, August 2017). No major pathology was detected associated with the GI trematodes in any cases, except for two cases with apparently unrelated GI disease (solitary intestinal fibro-necrotic granuloma presumably nematode-related, and a mixed bacterial gastroenteritis).

Three types of trematodes were detected in the urinary bladder from four turtle cases, including gorgoderid bladder flukes (*Plesiochorus cymbiformis* and *Plesiochorus* sp.) and flukes derived from the GI tract, including a pronocephalid (*Pyelosomum* sp.) and a paramphistome (*Schizamphistomum* sp.), none of which were associated with any major pathology.

Grossly trypanorhynch metacestodes were a very common finding, presenting as small, discrete, raised cream masses, and histologically as metazoan cysts, completely walled off and surrounded by a true histiocytic reaction (Figure 4.12). Trypanorhynch metacestodes were detected in the digestive system (including stomach, intestine and liver) of 48/61 (78.7%) cases examined (excluding D4 animals and cases with incomplete GI tract examination at necropsy). Individuals within all species and all age groups of sea turtles examined were observed to show evidence of this parasitic disease, except the single olive ridley turtle.

With support from expert parasitologists, one trypanorhynch in a flatback turtle was identified morphologically as a type of otobothriid (as evidenced by the bothrial pits), and speciated as *Parotobothrium balli*, due to the homomorphous and homeoacanthous hooks on the inverted tentacular armature (Emeritus Prof. I. Beveridge, University of Melbourne, personal communication, 16 May 2017) (Figure 4.13). Species was further confirmed by molecular techniques.

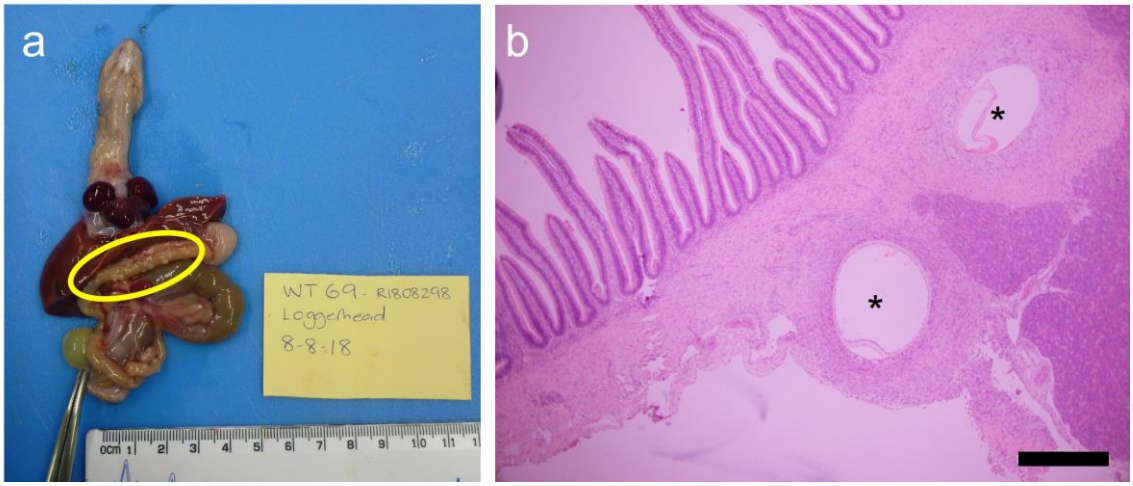


Figure 4.12 (a) Trypanorhynch metacestodes (larvae) seen as raised cream masses in the GI tract (yellow circle); and (b) Photomicrograph of a trypanorhynch cysts (asterisks) in the tunica muscularis of the small intestine (H&E, x40, scale bar 500µm).

Figure 4.13 Trypanorhynch metacestode from a flatback turtle identified as *Parotobothrium balli* (Photo credit A. Elliot, Parasitology Department, Murdoch University).

Two turtles, including juvenile hawksbill (WT7) and green turtles (WT26), had coccidia detected on faecal examination; possibly *Caryospora* sp. cysts in one sample (WT26), however no coccidia or related pathology were detected on histopathology.

Gastrointestinal impaction and marine debris ingestion

Ingestion of marine debris was found in 9/75 (12.0%) cases in this WA study. Marine debris was mainly found in the GI tract at necropsy, but in two cases foreign bodies were passed in care only, and in another case, marine debris was both passed during rehabilitation and found on necropsy. Marine debris ingestion was only assigned to be the cause of mortality in one case (WT29) where necropsy and histopathology detected ulceration and transmural necrotic gastro-enteritis associated with 343 pieces of marine debris (counted by Dr E. Duncan, Exeter University, 14 November 2019), including synthetic line, soft and hard plastic (Figure 4.14). Marine debris contributed to morbidity in one other case (WT71), with plastic and string detected in the impaction (Figure 4.14). Similar to the first case, this animal also had biochemistry changes (Section 4.3.5 – Ante-mortem associations with pathology), reduced defecation and inappetence reported after several weeks in care, but only a mild intestinal mucosal inflammation on histopathology. One other case (WT12) died from an iatrogenic impaction caused by inappropriate techniques during gastric tube feeding. One post-hatchling (WT51) also passed 119 pieces of marine debris (78.2% thread) but had no evidence of GI pathology. Another post-hatchling loggerhead (WT38) had a piece of hard plastic associated with an impaction in the distal colon but the primary cause of mortality was identified as endoparasitoses.

The majority of the marine debris detected in the GI tracts of cases in this study which underwent detailed classification was synthetic thread (8/17, 47.1%), most-likely from fishing-related activities (i.e., degraded fishing nets), followed by sheet plastic (6/17, 35.1%) (Dr E. Duncan, Exeter University, personal communication, 14 November 2019). Species and age-classes affected included juvenile green turtles, and post-hatchling loggerhead and flatback turtles (n=3 for each group).

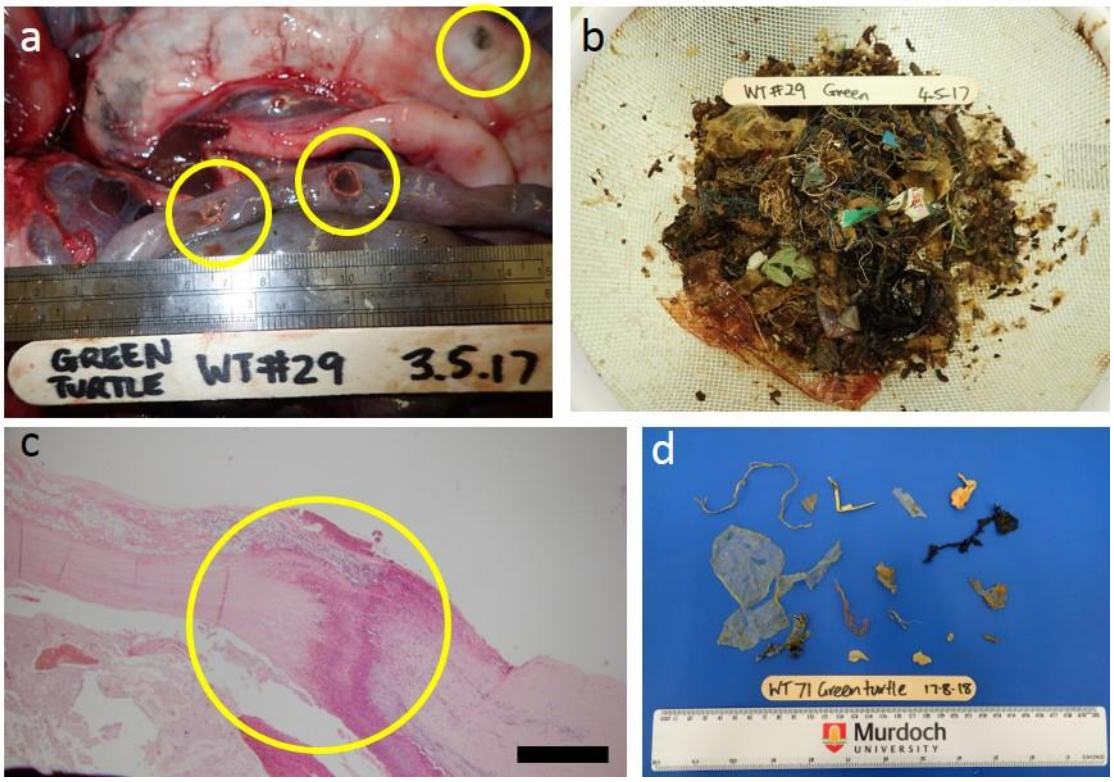


Figure 4.14 (a–c) Green turtle (WT29) showing (a) Ulcerated GI tract (yellow circles); (b) Marine debris found in GI tract; (c) Photomicrograph of transmural gastro-necrotic enteritis (yellow circle) (H&E, x40, scale bar 500 µm); and (d) Marine debris from green turtle (WT71) with GI impaction contributory to mortality.

While ileus is a non-specific sign of disease, when we excluded healthy turtles which had died from acute trauma, other turtles with full GI tracts (n=5), possibly related to ileus, were found to have diseases affecting the GI (spirorchiidiasis), neurological (spirorchiidiasis), hepatobiliary (trematodiasis), and renal (bacterial nephritis) body systems. However, due to autolysis/freezing, subtle pathology associated with ileus, such as oedema, was difficult to detect on histopathology.

Systemic inflammation

On necropsy, signs of systemic inflammation in stranded sea turtles were evidenced by a coelomitis in 15/75 (20.0%) cases (including cases which caused mortality and those which contributed to mortality), with fibrinous strands throughout the coelomic cavity. Histopathology usually showed coelomitis; often accompanied with hepatic

capsulitis, which presented with a range of other features including heterophilic inflammation, fibrin deposits and necrosis.

Osmoregulatory disorder

When the salt glands of the stranded sea turtle cases were examined on necropsy, two cases (2/75, 2.7%) had salt gland adenopathy (WT8 and WT31), evidenced by accumulation of inspissated material in the salt glands. On histology, both showed cellular changes, including metaplasia and hyperplasia, and ductular obstruction, and one turtle (WT8) also had necrotic bacterial adenitis.

Gout/Pseudogout

During examination of sea turtle cases at necropsy, one case presented with gout/pseudogout. This case (WT39) presented with tophaceous deposits throughout the visceral organs including liver, spleen, kidney, heart as well as muscle, bronchi and brain, and white liquid through to pasty material within the joint spaces of the limbs and vertebrae (Figure 4.15). When a sea turtle case presents with this type of pathology, it is important to distinguish between true gout, pseudogout and oxalosis. In true gout, deposits are uric acid; in pseudogout, deposits are calcium pyrophosphate deposits; and in oxalosis, deposits are calcium oxalate crystals. A range of laboratory testing was performed, including Murexidine, Pizzolato's, silver, Alizarin Red, lithium carbonate extraction-hexamine silver, and non-aqueous alcoholic eosin (bifringence test) stains. Together, the results from these tests were positive for pseudogout, inconclusive for gout, and negative for oxalosis. This turtle also displayed periorbital swelling, as well as swelling in a single joint (right tarsometatarsal joint). A lack of mineralisation and radiodensity on radiography was consistent with a diagnosis of gout (Perpinan 2017). Uric acid was also highly elevated (1.749 mmol/L), and calcium was within normal limits (1.78 mmol/L). While uric acid may be increased with both gout and pseudogout, an increase in calcium would be expected with pseudogout but not with gout. Further, while both gout and pseudogout can affect the joints, soft tissue deposits would be expected with gout but not with pseudogout.

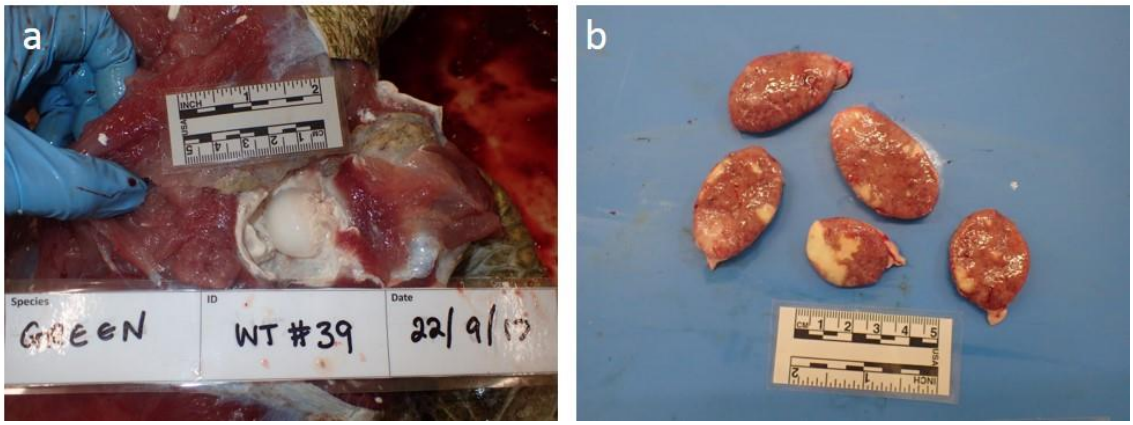


Figure 4.15 Gross pathology of green turtle (WT39) with (a) Articular gout/pseudogout of the joint; and (b) Visceral gout of the spleen (serially sectioned) with necrosis due to gout tophi.

Nephritis

On examination of the urogenital system of stranded sea turtle cases at necropsy, there were two cases of bacterial nephritis. One of the two cases (WT17) had a unilateral renomegaly with abscesses associated with bacteria comprising heterophilic, necrotic granulomas, surrounded by fibrosis. The second case (WT35) had an ascending bacterial urinary tract infection due to a tail injury affecting the cloacal opening, resulting in bilateral tubular necrosis and interstitial nephritis and a proximal ureteral obstruction and ulcerative ureteritis. Microbiological culture reported a mixed bacterial growth (*S. iniae* excluded). This turtle (WT35) also had a fibrino-necrotic and suppurative serosal cystitis and coelomitis. The first case (WT17) also had bilateral pneumonia with bacteria (Gram-positive diplococci or cocci in short chains, possibly *Enterococcus* spp.) and fungi associated; but as this was a frozen cadaver no microbiology was performed, we were unable to determine if the micro-organisms associated with the pneumonia were related to the bacteria associated with the nephritis.

Miscellaneous

Myopathy (protozoal and general)

A number of the stranded sea turtle cases had some form of myopathy. One case (WT13) with a moderate myopathy had pale and streaky skeletal muscle grossly; and histologically there was evidence of acute rhabdomyopathy associated with a micro-organism (Figure 4.16). Calcofluor stains were performed and fluoresced on fluorescence microscopy. Electron-microscopy was utilised to identify this organism as a microsporidium; a fungal organism (Figure 4.16) (M. Platten, DPIRD, personal communication, 4 December 2018). PCRs for the microsporidia and pan-fungal PCRs, were performed at the Reptile Pathogen Laboratory Murdoch University (Dr T. Hyndman, Murdoch University, personal communication, 22 July 2019). Both PCRs were negative, and the species was unable to be identified.

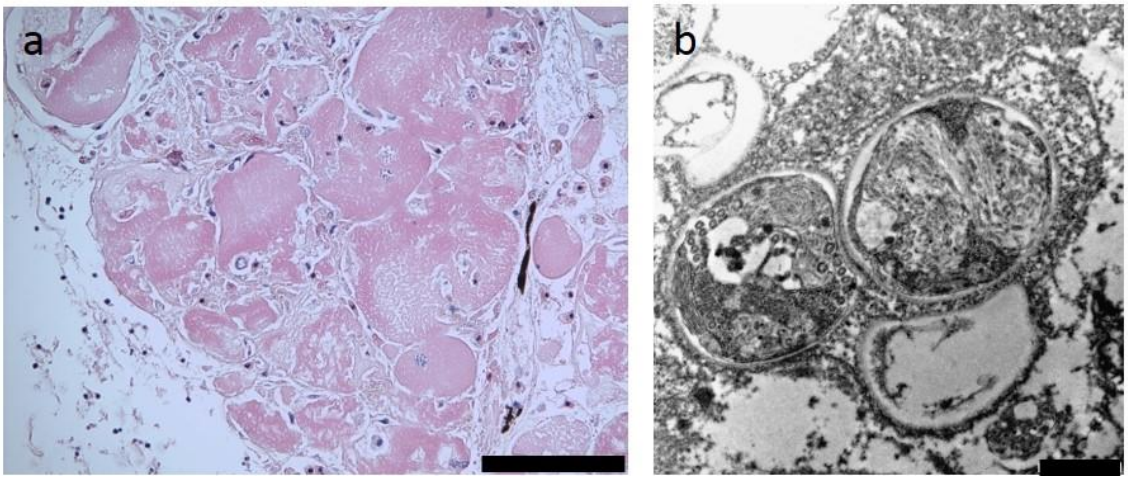


Figure 4.16 (a) Photomicrograph of fungal pseudocysts in the muscle accompanied with acute rhabdomyopathy (H&E, x100, scale bar 200 µm); and (b) Electron microscopy of the fungal organism (scale bar 1 µm).

In other cases, myopathy was evidenced occasionally by gross muscle changes. On histology, there was evidence of acute to peracute degeneration, necrosis, and vacuolation of myocytes, accompanied occasionally by fibrosis and myositis (Section 4.3.5 – Ante-mortem associations with pathology).

4.3.5 Data analysis

Health status

Health status of stranded sea turtle cases was assigned using the combined results from the various diagnostic tests. Of the stranded turtle cases, 55/75 (73.3%) were assigned as 'unhealthy', 3/75 (4.0%) were assigned as 'healthy', and the remaining 17/75 (22.7%) cases were assigned as 'unknown' health status. As expected, all healthy cases were in good body condition and had died from an acute trauma. Due to the small sample size of the healthy group, statistical testing was not performed.

Stranding survey

Using only data from the stranding survey (i.e., no veterinary diagnostics performed), a preliminary cause of stranding was assigned in 67/75 (89.3%) cases (Figure 4.17). From the survey, the most common generic cause of standing was found to be chronic debilitation (21/75, 28.0%). Trauma accounted for 15/75 (20.0%); noting six of these were recategorized (Section 4.3.4). Cold-stunning accounted for 14/75 (18.7%) strandings, all of which were post-hatchling loggerhead turtles found during winter in south-western WA (south of latitude -31.5038). Eleven post-hatchling flatback turtles were part of a mass mortality event associated with *S. iniae* (11/75, 14.7%) (Chapter five). Of the 11 live turtles found floating in the water, the primary cause of stranding was assigned as buoyancy disorder in 3/11 (27.3%) floating cases – accounting for 3/75 (4.0%) the total stranded turtle cases. Floating was also present in turtles categorised with chronic debilitation (6/11, 54.5%), entanglement (1/11, 9.1%) and trauma (1/11, 9.1%). A total of two cases were assigned entanglement (2/75, 2.7%) and one as fibropapillomatosis (1/75, 1.3%). The cause of stranding could not be determined in the remaining eight cases assigned unknown category (8/75, 10.7%).

Morbidity and mortality investigations

The most likely final cause of mortality was determined in 62/75 (82.7%) stranded sea turtle cases, through ante-mortem and/or post-mortem veterinary diagnostics (Figure

4.18). Spirorchiidiasis was the most common primary cause of mortality in this study, with an incidence of 24/75 (32.0%). Of the total spirorchiidiasis cases, systemic spirorchiidiasis accounted for 19/24 (79.2%), and neurospirorchiidiasis accounted for 5/24 (20.8%), with some overlap of the two forms of the disease in these spirorchiidiasis cases. The next most common cause of mortality was identified to be trauma, which accounted for 10/75 (13.3%) total mortality cases. Of the total trauma cases, anthropogenic causes accounted for 7/10 (70.0%), natural predation accounted for 1/10 (10.0%), and unknown trauma accounted for 2/10 (20.0%). Causes of anthropogenic trauma included vessel strike and customary take (3/75, 4.0% for each) and entanglement in marine debris (1/75, 1.3%). Other examples of anthropogenic causes of mortality observed in this study were marine debris ingestion and iatrogenic GI impaction (1/75, 1.3% for each).

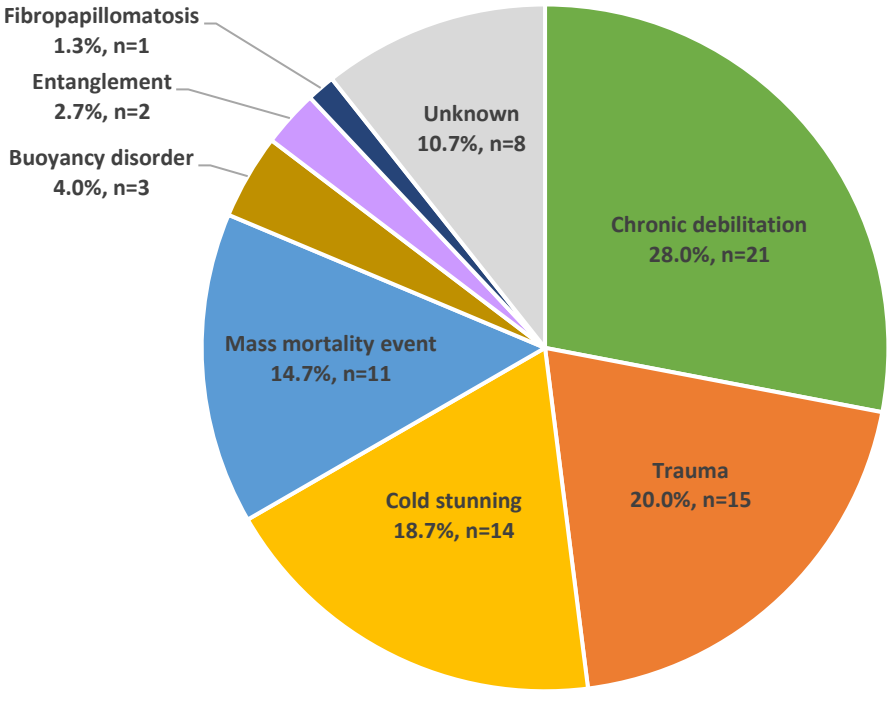


Figure 4.17 Causes of standing as identified from the stranding survey for each of the eight stranding categories, as a percentage of the total study animals (n=75) (see Appendix 18).

Aside from spirorchiidiasis, this study found another disease that was responsible for a relatively high proportion of total mortality cases: endoparasitosis. Endoparasitoses accounted for 8/75 (10.7%) of all mortality cases. Of the total cases of endoparasitoses, GI cestodiasis accounted for 3/8 (37.5%), mixed GI endoparasitosis (nematodes, cestodes and trematodes) accounted for 2/8 (25.0%), unknown GI endoparasitosis accounted for 2/8 (25.0%) and there was a single case of cholecystic trematodiasis (1/8, 12.5%).

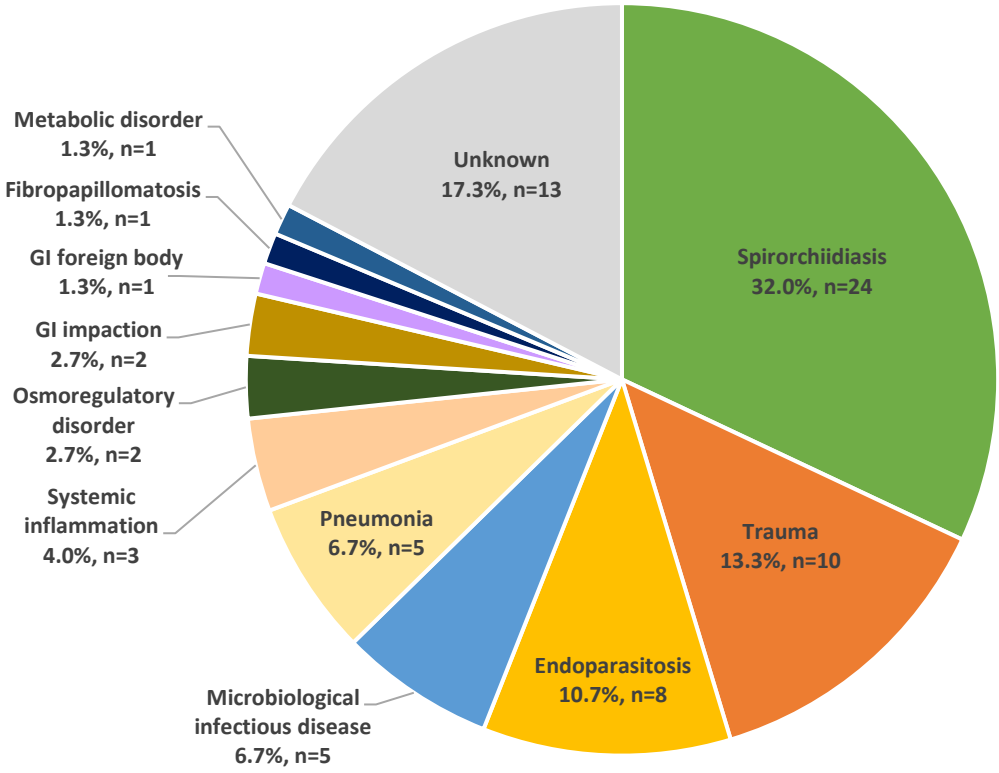


Figure 4.18 Final cause of mortality showing the percentage for each of the 12 mortality categories of the total stranded sea turtle mortality cases (n=75) (see Appendix 18).

Another disease-related primary cause of death identified in this study was microbiological infectious disease, which accounted for 5/75 (6.7%) of total mortality cases. Of the total microbiological infectious disease cases, an infectious agent (*S. iniae*) was able to be identified in 3/5 (60.0%) cases and the remainder were assigned to unknown bacteria (2/5, 40.0%). Cases with coelomitis where no agent was detected

were assigned systemic inflammation for the cause of mortality category (3/75, 4.0%). The proportion of total mortality cases identified to have had pneumonia as a primary cause of death was 5/75 (6.7%), including four cases of verminous pneumonia and one case of unknown aetiology. Five remaining disease categories (including osmoregulatory disorder, GI impaction, GI foreign body, fibropapillomatosis and metabolic disorder) also had a small number of cases. Cause of mortality was unknown in 13/75 (17.3%) of total mortality cases, making this the second largest category.

When assessing body systems of total mortality cases affected by disease-related deaths (including both infectious and non-infectious disease which includes trauma), the most common cause of death was systemic disease (26/75, 34.7%), followed by digestive system disease (10/75, 13.3%), neurological system disease (8/75, 10.7%), musculoskeletal system disease (6/75, 8.0%) and respiratory system disease (5/75, 6.7%), with unknown body system disease accounting for 13/75 (17.3%).

When attempting to reach a diagnosis for final cause of mortality for the stranded sea turtle cases, and when considering the level of autolysis of cases, a diagnosis was (as expected) more likely in turtles with minimal autolysis (D1 and D2) (Fisher's exact test OR=27.9, 95% CI: 4.88-300.73, $p<0.001$). A diagnosis was able to be made in 54/56 (96.4%) cases which had minimal autolysis, whereas for decomposed cadavers, a diagnosis was able to be made in only 9/19 (47.4%) cases. All deceased rehabilitation cases (both fresh and freshly frozen) were able to be assigned a final cause of death diagnosis. When reviewing 'final cause of death' diagnoses by rehabilitation status, we obtained the following data for the various 'final cause of death' categories. Where spirorchiidiasis was assigned as the final cause of death, this was assigned for 12/34 (35.3%) rehabilitated cases, and 12/41 (29.3%) non-rehabilitated cases. Where pneumonia was assigned final cause of death, this was assigned for 4/34 (11.8%) rehabilitated cases, while there were no reports of pneumonia in non-rehabilitated turtles. Where endoparasitosis was assigned as the final cause of death, this was assigned for 5/34 (14.7%) rehabilitated cases, and 3/41 (7.3%) non-rehabilitated cases. Where trauma was assigned as the final cause of death, this was assigned for 2/34

(5.9%) rehabilitation cases, and 8/41 (19.5%) non-rehabilitated cases. When comparing body condition of rehabilitation and non-rehabilitation cases, 24/35 (68.6%) rehabilitated turtles were in poor or emaciated body condition (low BCS), while for non-rehabilitated turtles 5/11 (45.5%) had a low BCS and 6/11 (54.5%) had a high BCS.

Multifactorial contributory causes to mortality

By examining only the stranded sea turtle cases in the contributory study group (n=54), (which excludes cases for which the contribution of disease to morbidity and mortality could not be established), we were able to report the frequency of all significant pathology and diseases detected, along with the prevalence of co-morbidities. It should be noted that in addition to the primary cause of mortality, some cases were found to have multiple diseases or disorders contributing to mortality (Table 4.8).

Body condition of mortality cases

When examining body condition of the stranded sea turtle cases for the different mortality categories, within the spirorchiidiasis study group, all the mortality cases for which spirorchiidiasis was identified as the primary cause of death were in poor body condition (BCS 1.5-2) or emaciated body condition (BCS 0.5-1) (total cases with low BCS, n=17). The exception was one case in long-term care. For the cases where neurological spirorchiidiasis was identified as the primary cause of death (i.e., only the neurological system had a severe rating), all were in average (BCS 2.5-3) or good body condition (BCS 3.5-4.5) (total cases with high BCS, n=4); except for one case in poor body condition, which presented with a chronic open flipper fracture. For the mortality cases where trauma was identified as the primary cause of death, all cases were in average or good body condition (total cases with high BCS, n=9), except one turtle with a chronic traumatic carapace injury. All cases with pneumonia (n=5) and salt gland adenopathy (n=2) were assigned emaciated or poor body condition, as were all endoparasitosis cases (n=8), and systemic inflammation cases (n=3), aside from one in each group. A low BCS was also assigned to a single case in each of GI impaction, GI

ulceration from marine debris ingestion, and bacterial nephritis categories, as well as two cases of unknown cause of mortality.

When considering body condition for the remaining mortality cases, all cases were in average or good body condition. This included cases with the final cause of mortality diagnosed as microbiological infectious disease (n=3), as well as a single case of each of the four disease categories identified as the primary cause of mortality (gout/pseudogout, fibropapillomatosis, GI impaction and bacterial nephritis; all of which were in mid-long term care). All cases in the unknown mortality category (n=10) also had a high BCS.

Disease duration of mortality cases

When examining disease duration of stranded sea turtle mortality cases by cause of mortality, for cases for which spirorchiidiasis was the primary identified cause of death, 21/24 (87.5%) were chronic, 2/24 (8.3%) were subacute, and 1/24 (4.2%) were acute. Of these total spirorchiidiasis cases, neurological spirorchiidiasis (5/24, 20.8%) accounted for all subacute and acute cases, and for a single chronic case. For mortality cases for which trauma was the primary identified cause of death, the time course of trauma was considered acute in 5/10 (50.0%) cases, chronic in 4/10 (40.0%) cases, and subacute in 1/10 (10.0%) cases. With respect to the time course of other diseases identified as the primary cause of death, the time frame for endoparasitosis was considered subacute in 6/8 (75.0%) cases, and chronic in 2/8 (25.0%) cases; all systemic inflammation cases (n=3) were considered subacute; all microbiological infectious disease cases (n=4) were considered acute; all pneumonia cases (n=5) were considered subacute; all cases of GI impaction or ingestion of marine debris (n=3) were considered chronic; all salt gland adenopathy cases (n=2) were considered chronic; and the single case of fibropapillomatosis was also considered chronic. For the remaining diseases, the time frames for bacterial nephritis included a single case each of chronic and subacute presentation, while a single case of metabolic disease was acute in presentation. The rest of the cases were of unknown cause (n=12), and therefore the time frame of disease was not reported.

Table 4.8 Diseases contributing to mortality and the body system involved, noting the contributory column includes both mortality and contributory causes of mortality. Contributory disease percentage represents disease proportion of the total contributory diseases reported (n=244), while contributory body system percentage represents the proportion of each body system.

System	Anatomical location	Disease	Mortality (n=54)	Contributory (n=54)	% Contributory disease	% Contributory body system
Generalised						11.1%
		Systemic spirorchiidiasis	15	22*		
		Systemic inflammation	3	15	6.1%	
		Gout/Pseudogout	1	1	0.4%	
		Cachexia		8	3.3%	
		Electrolyte derangements		3	1.2%	
Musculoskeletal						6.1%
	Shell	Trauma	2	4	1.6%	
	Head	Trauma	3	3	1.2%	
	Limbs	Trauma	2	6	2.5%	
	Tail	Trauma		1	0.4%	
	Muscle	Fungal myopathy		1	0.4%	
Respiratory						12.3%
	Lungs	Spirorchiidiasis	12*	14	5.7%	
		Pneumonia	5	14	5.7%	
		Drowning		1	0.4%	
		Pulmonary fibropapillomatosis		1	0.4%	
Cardiovascular						9.4%
	Heart and great vessels	Spirorchiidiasis	14*	21	8.6%	
		Cardiomyopathy		2	0.8%	
Digestive						14.3%
	Gastrointestinal	Spirorchiidiasis	13*	17	7.0%	
		Cestodiasis	3	6	2.5%	
		Mixed GI endoparasitosis	2	2	0.8%	
		Unknown endoparasitosis	2	2	0.8%	
		Nematodiasis		2	0.8%	
		Impaction	2	2	0.8%	
		GI Foreign body	1	2	0.8%	
		Bacterial gastroenteritis		1	0.4%	
		Cloacal trauma		1	0.4%	
	Liver and gall bladder	Spirorchiidiasis	5*	7	2.9%	4.9%
		Cholangio-cholecystitis (parasitic)	1	5	2.0%	

System	Anatomical location	Disease	Mortality (n=54)	Contributory (n=54)	% Contributory disease	% Contributory body system
Urogenital						5.7%
	Kidneys	Spirorchiidiasis	8*	12	4.9%	
		Bacterial nephritis	2	2	0.8%	
Lacrimal						6.1%
	Salt glands	Spirorchiidiasis	9*	13	5.3%	
		Adenopathy	2	2	0.8%	
Endocrine						5.3%
	Thyroid	Spirorchiidiasis	10*	13	5.3%	
Neurological						7.8%
	Brain	Spirorchiidiasis	5 (12*)	19	7.8%	
Integumentary						2.0%
	Skin	Fibropapillomatosis	1	2	0.8%	
		Traumatic ulcerative dermatitis	1	3	1.2%	
Sensory						
	Eye	Ocular fibropapillomatosis		1	0.4%	0.4%
Unknown			1	1	0.4%	0.4%
None			0	14	5.7%	5.7%
Total			54	244	100%	100%

* Not included in count. For example, for cases with neurospirorchiidiasis or systemic spirorchiidiasis as the primary cause of mortality, systemic spirorchiidiasis or neurospirorchiidiasis was included in the primary cause of mortality count or contributory cause of mortality count, but not both. For other cases with systemic spirorchiidiasis as the primary cause of death, numbers presented show body systems affected by systemic spirorchiidiasis not included in count to avoid duplication of counts.

Disease duration was found to be significantly correlated with season. Specifically, chronic disease was correlated significantly with the cool season (p=0.004), and in the cool season, chronic disease was 1.3 times more likely than acute/subacute disease (OR=5.85, 95% CI: 1.85-22.75, p=0.005).

Multiple diseases in mortality cases

All sea turtle mortalities in this study, even apparently healthy turtles, had evidence of multiple disease processes (even if this was a solitary pulmonary granuloma or mild-moderate spirorchiidiasis burden). When considering whether the observed pathology was contributory to mortality, 39/54 (72.2%) cases in the contributory study group

were identified to have multiple diseases contributing to mortality; in the remaining 15/54 (27.8%) cases in the contributory study group, a single disease (including non-infectious diseases such as trauma and marine debris ingestion) were identified as the likely cause of death. Single causes of death include spirorchiidiasis (8/15, 53.3%), trauma (6/15, 40.0%) and metabolic disease (1/15, 6.7%).

When considering which diseases occurred together, typically endoparasitoses, occurred together with systemic inflammation (coelomitis) or pneumonia as co-morbidities; while pneumonia (n=4), endoparasites (n=3) and trauma (n=3) occurred commonly with spirorchiidiasis.

When examining if multiple diseases were more likely to occur with disease chronicity, chronic disease was not significantly more likely to occur when compared with acute/subacute disease. However, when data from chronic disease and subacute disease cases were pooled, chronic/subacute disease was 3.36 times more likely when multiple diseases were present (OR=6.73, 95% CI: 1.16-53.48, p=0.041).

Infectious disease

When examining infectious disease responsible for sea turtle mortality in this study, it should be noted that 20/75 (26.7%) mortality cases were classed as 'unknown' cause. Infectious disease was identified to be responsible for a minimum of 41/75 (54.7%) of the sea turtle mortalities in this study. Another 33/75 (44.0%) mortality cases were identified to be due to parasites, and 24/75 (32.0%) were identified to be due to spirorchiidiasis. Bacteria were identified to account for 7/75 (9.3%) cases, and viruses only 1/75 (1.3%). Non-infectious disease was the identified cause of mortality in 15/75 (20.0%) cases.

If examining only the contributory study group (n=54) and considering only the primary cause of mortality, the primary cause of mortality was identified to be infectious disease for 34/54 (63.0%) cases and non-infectious disease for 12/54 (22.2%); the remaining 8/54 (14.8%) were classed as unknown cause. If expanding to include all diseases that were identified to contribute to mortality, an additional 40

different additional diseases were identified (in n=32 cases; some cases had multiple diseases). This included 28 additional infectious diseases and 12 additional non-infectious diseases, resulting in a total of 62 different infectious diseases reported (62/86, 72.1%), and 24 different non-infectious diseases (24/86, 27.9%). Of the infectious disease group, 46/86 (53.8%) were parasites, 7/86 (8.1%) were bacteria, 5/86 (5.8%) were fungal diseases, and 4/86 (4.7%) were viral diseases. When including both the primary cause and contributory causes of mortality (i.e., including some cases for which the disease agents in the primary cause of death were 'unknown', but for which the agents responsible for contributory disease were known), together these diseases contributed towards mortality of the total contributory study group i.e., 54 animals.

Additional testing for the infectious agent mycobacteria was performed using ZN acid-fast stain in pneumonia cases, however, in the four cases tested, no mycobacteria were detected.

Anthropogenic vs natural causes of mortality

For the mortality cases in this study, direct anthropogenic causes of mortality included vessel strike (3/75, 4%), entanglement (1/75, 1.3%) and marine debris ingestion (1/75, 1.3%). Indirect anthropogenic causes of mortality, for example toxicity and habitat degradation leading to food shortages and starvation, are difficult to identify. In this study, when considering the primary identified causes of mortality, 9/75 (12.0%) mortalities were identified to be due to direct anthropogenic causes. When considering causes contributory to mortality, anthropogenic causes were found to contribute to 12/75 (16.0%) mortalities (one for each category of vessel strike, entanglement and marine debris ingestion). Natural causes of mortality were identified to be responsible for 52/75 (69.3%) mortalities. The causes of mortality for the remaining mortalities were unknown (14/75, 18.7%); indicating that the previous figures regarding anthropogenic and natural causes of death are minimums only.

Ante-mortem associations with pathological and parasitological findings

Of the turtles which received a clinical examination (n=31), seven had neurological signs (7/31, 22.6%). Of these seven, the cause of mortality was severe neurospirorchiidiasis for three cases (3/7, 42.9%), two were cold-stunned and had systemic inflammation and pneumonia (+/- endoparasitoses) (2/7, 28.6%), one had head trauma (1/7, 14.3%), and one had a GI impaction (1/7, 14.3%). Of the 24 neurologically normal turtles, four had neurospirorchiidiasis (4/24, 16.7%), four presented with trauma (including one head trauma) (4/24, 16.7%), 10 were cold-stunned (10/24, 41.7%), and the remaining six had various diseases (6/24, 25.0%) including ingestion of marine debris.

Of the turtles which had a clinical examination (n=31), three were reported to have respiratory signs (3/31, 9.7%), including one bacterial pneumonia case and two cases of endoparasitosis. Ten other cases were identified to have pneumonia or other pulmonary disease (e.g., FP pulmonary tumour) contributing to mortality (10/31, 32.2%), and for these, no respiratory signs were detected.

Of the 27 live turtles which were assessed for buoyancy either through a clinical examination (21/27, 77.8%) or were reported floating at the time of stranding (6/27, 22.2%), 24 had abnormal buoyancy (24/27, 88.9%), likely associated with a variety of identified systemic diseases, including spirorchiidiasis, GI endoparasitosis, trauma and pneumonia. The cases with normal buoyancy (4/27, 14.8%) were identified to have pneumonia, bacterial nephritis or metabolic disease.

Nine sea turtles which had clinical examinations also had blood and muscle histology performed (9/31, 29.0%). Of these, five were found to have moderate-to-marked increases in CK (>7500– 55,000U/L) +/- AST (5/9, 55.6%), but when these findings were correlated with muscle pathology, only two showed moderate myopathy (2/9, 22.2%). Of the four turtles with normal CK (4/9, 44.4%), one had evidence of mild muscle pathology (1/9, 11.1%). The cases with the highest CK were two cold-stunned turtles

(WT69 and WT61), a single case of ingestion of marine debris (WT29), and a single case of entanglement in marine debris (WT40).

Of the stranded turtle cases which had clinical examinations performed, cases with severe systemic inflammation, as evidenced by severe leucocytosis (usually accompanied with heterophilia), included an entanglement case with ulcerative dermatitis (WT40); a neurospirorchiidiasis case (WT46); a marine debris ingestion case with necrotic-ulcerative gastro-enteritis (WT29); and a turtle with bacterial nephritis due to cloacal trauma (WT35). Anaemia was detected in one amputation case (WT48).

Another stranded turtle case which had a clinical examination and which, during post-mortem investigations, was diagnosed with gout/pseudogout (WT39), had extremely elevated uric acid (1.749 mmol/L), hyperglycaemia (16.1 mmol/L), moderately high CK (8000 U/L), high BUN (80 mmol/L), as well as elevated bile acids (significance unknown).

Other stranded turtles which had clinical examinations performed, including the marine debris ulcerative gastroenteritis case (WT29) and the entanglement ulcerative dermatitis case (WT40), were found to have severe electrolyte disturbances (i.e., sodium >180 mmol/L and chloride >130 mmol/L). Both these cases also showed evidence of dehydration (haemoconcentration), hyperuricaemia and hyperphosphataemia. Only the marine debris ingestion case (WT29) had increased BUN, and only the entanglement case (WT40) had hyperglycaemia, which was likely stress related.

It should be noted that several other turtles which had blood collected after the five day cut-off had major blood abnormalities; including one case with severe spirorchiidiasis (WT26) with anaemia, leucocytosis, low Ca: P and high BUN, CK and AST; one GI impaction case with contributions by marine debris (WT71) had electrolyte derangements, low Ca: P and high AST; and one case with severe spirorchiidiasis (WT54) had severe hyponatraemia (which was housed in freshwater for over two weeks).

Of the clinical stranded turtle cases which received praziquantel treatment for spirorchiidiasis, only three turtles (3/7, 42.9%) received a complete praziquantel course for spirorchiidiasis. Of these three, all were diagnosed with spirorchiidiasis, as either the primary cause of mortality or contributing to mortality; however no adult parasites were detected in any of these cases. Two of the three cases were from rehabilitation (WT32 and WT33) and had been treated with a parasiticide approximately two years prior to death and necropsy. While it is possible that spirorchiid eggs and associated pathology remained despite the effective treatment of adult spirorchiids, these turtles were kept in sea water tanks; and there is therefore potential for intermediate hosts to be present, which would complete the life cycle. The turtle with severe cardiovascular spirorchiid pathology (WT32) appeared to have a primary site of egg deposition (>100 eggs) in the right aorta; this sea turtle had either been infected prior to being taken into rehabilitation and survived for an extended period before succumbing to the disease, or was infected during the time housed in the aquarium and the parasites were not detected through post-mortem diagnostics. The other case (WT46) was frozen prior to necropsy, impacting the quality of the results and the ability to detect live adult spirorchiids.

With regards to the clinical stranded sea turtle cases which received other medical treatments, of the turtles which completed an antimicrobial course (6/14, 42.9%), one had an infectious disease (bacterial pneumonia) (1/6, 16.7%). For the eight cases which received an incomplete course of antimicrobials, one was found to have a bacterial nephritis, and no microbial agents were detected in the other cases.

Associations between cause of stranding and final cause of mortality

When comparing the 'cause of stranding' category and the final identified 'cause of mortality' category for each stranding case, it was found that chronic debilitation (cause of stranding category) was attributed to spirorchiidiasis (cause of mortality category) in 16/21 (76.2%) cases. When comparing the remaining cases of chronic debilitation (cause of stranding category) with the cause of mortality category, the salt gland adenopathy category accounted for two cases, and each category of marine

debris ingestion, GI impaction and endoparasitosis (specifically trematode cholecystitis) accounted for a single case.

When cold-stunned turtles in the cause of stranding category were compared with those in the cause of mortality category, all were diagnosed with diseases related to altered immunity, including endoparasites (7/14, 50.0%), pneumonia (5/14, 35.7%) and coelomitis (2/14, 14.3%).

All cases for which the final cause of mortality was reported to be trauma (10/75, 13.3%) also had trauma assigned as the cause of stranding, except for one case for which the assigned cause of stranding was entanglement, and which died as a result of traumatic skin injuries (+/- infection) associated with entanglement. There were seven other stranding cases for which their stranding category was trauma or entanglement, and for these cases, final cause of death was identified to be either spirorchiidiasis, bacterial nephritis (related to a tail injury), fibropapillomatosis, or unknown. Of this list, there were two spirorchiidiasis cases; one of which (WT18) was likely to have suffered a vessel strike secondary to severe spirorchiidiasis, while the other (WT50) had severe neurospirorchiidiasis and missing limbs (although it was unclear if the missing limbs were due to trauma, scavenging, or both). When examining stranded turtle cases with external abnormalities likely be related to cause of stranding (n=17; 15 trauma and two entanglement cases), trauma was able to be assigned as a contributory cause of mortality in 12/17 (70.6%) cases. Conversely, there were an extra four mortality cases where trauma was considered contributory to mortality, for which their stranding category was assigned as either cold-stunning and chronic debilitation.

Of the 11 cases from a mass mortality event, only three were confirmed as having *S. iniae*, leaving the cause of mortality for the remaining eight cases as ‘unknown’.

Importantly, with one exception, all stranding cases for which the cause of stranding was initially recorded as ‘unknown’, were able to have likely causes of stranding attributed once cause of death was determined. These included spirorchiidiasis (4/8,

50.0%; including two cases of neurospirorchiidiasis), and one case each of coelomitis, gout/pseudogout and bacterial nephritis (1/8, 12.5% for each group).

When comparing buoyancy disorder (cause of stranding category) with the cause of mortality category, a single case was assigned for each of the GI impaction, neurospirorchiidiasis and unknown categories.

4.4 Discussion

Through multi-modal disease investigation techniques, primarily necropsy, histopathology and parasitology, this project has established the first baseline for causes of morbidity and mortality of stranded sea turtles in WA.

The primary identified cause of mortality was natural disease (69.3%). Direct anthropogenic threats were identified as causing 12.0% of mortalities; although with the caveat that 18.7% of mortality cases were assigned an unknown cause. These findings support those by Raidal et al. (1998) who reported that direct anthropogenic impacts were low for sea turtles in WA. In other Australian studies, direct anthropogenic causes of sea turtle mortalities have been reported at 7.2% of total mortalities assessed (Flint et al. 2010d) through to 34% (Gordon 2005); although in both cases, these figures would be a minimum, given that some mortalities were assigned an unknown cause. In other parts of the world, anthropogenic mortalities are often reported to be much higher, for example 71.2% of sea turtle mortalities examined in the Mediterranean were found to have anthropogenic causes (Oros et al. 2016).

Similar to findings from other sea turtle health studies, the natural diseases identified in the sea turtles in this study were primarily infectious (62/86, 72.1%) (Glazebrook and Campbell 1990a, Raidal et al. 1998) and dominated by parasites (46/62, 74.2%) (Tagliolatto et al. 2020), most of which were spirorchiids (30/46, 65.3%) (Flint et al. 2010d).

Spirorchiidiasis was the leading cause of mortality in this WA study (32.0%) including all five species examined (green, loggerhead, olive ridley, hawksbill and flatback turtle). The high prevalence of spirorchiidiasis in applicable cases (i.e., the spirorchiid study group), at 93.2%, was not unexpected, considering previous reports in this state (Raidal et al. 1998, Raidal et al. 2006) and elsewhere in Australia (Gordon 2005, Flint et al. 2010d).

This study's findings have contributed to filling regional, species and disease knowledge gaps, by establishing a baseline for causes of disease and mortality of sea turtles in WA and the east Indian Ocean. We reported spirorchiidiasis in flatback turtles for the first time (Wildlife Health Australia [WHA] electronic Wildlife Health Information System [eWHIS], accessed 21 November 2017). This study also found evidence of pathogenic bacteria not previously described in reptiles – *S. iniae* – associated with a mass mortality event (Chapter five); and reported the occurrence of diseases that are considered unusual in sea turtles, including gout/pseudogout and microsporidial myopathy.

4.4.1 Stranding survey

From the data on stranded turtles in this study, the most common initial cause of admission was chronic debilitation (21/75, 28.0%). Information obtained from the external necropsy examination was essential in identifying these cases. However poor body condition was correctly reported on the stranding form in only four cases (4/6, 66.7%). Further, when considering association of BCI with body condition of stranded sea turtles, for green turtles in our study that were within the size range studied previously (i.e., 30-80 cm), BCI corresponded well with the body condition for these turtles. However, there was poor association of BCI with body condition of turtles in the post-hatchling age-class, such as post-hatchling loggerheads. It was unclear whether this lack of association was a result of age-class and species, or age-class alone, as there were no green post-hatchling turtles in this study to determine whether BCI and body condition were associated.

There may be several reasons why this study did not observe sea turtles which die from acute causes. Firstly, turtles can often survive with major injuries (Aguirre and Lutz 2004) and diseases, and are more likely to strand late in the course of disease (Gordon 2005). Secondly, chronically unwell turtles are also more likely to present, compared to cases which die from acute issues such as vessel strike and fisheries-related drowning; the latter cases generally sink and are not located until the carcass putrefies and resurfaces, only later potentially arriving at the shore (usually in advanced decomposition) (Epperly et al. 1996). This makes identifying cause of death particularly challenging, especially for fisheries interactions, where there is often a lack of circumstantial evidence i.e., presence of fishing gear or external marks (Stacy et al. 2017a) which likely means that the impact of fisheries interactions on sea turtles is underestimated (Chaloupka et al. 2008, Casale et al. 2010). This may also explain why by-catch and drowning (also difficult to confirm) were not reported in our study. There may also be other reasons why this study did not observe sea turtle deaths that could be attributed to fishing interactions. For example, while harmful fisheries interactions have been reported for leatherback turtles in WA (Prince 2007), it is possible that there are fewer fisheries in sea turtle geographic ranges in WA compared with other locations in Australia and overseas (Orós et al. 2005, Flint et al. 2010d, Oros et al. 2016). As well, local oceanic currents may reduce the likelihood that turtles involved in fisheries interactions in WA are washed up along the coast of WA, especially considering only an estimated 7-20% of turtles that die in coastal areas are recovered (Epperly et al. 1996, Stacy et al. 2017a). This highlights the importance of collaboration between researchers, the state Fisheries Department and the fisheries industry, to increase the likelihood that by-catch turtles are reported; as well as the importance of collaboration with other industries and ports where sea turtles may be injured, entrapped and die suddenly.

While chronic debilitation in sea turtles has been reported previously in Australia (Flint et al. 2009b), cold-stunning syndrome has generally been unreported in mortality studies in Australia. The reports of cold-stunning syndrome in our study are likely related to the inclusion of higher latitude areas in our study than in most previous

Australian studies. Reports of cold-stunning cases are also determined by the number and intensity of autumn and winter storm fronts which blow turtles onshore. The majority of these cases occurred in 2017 in which many cold-stunned post-hatchling turtles were reported. Similar numbers may have occurred in other years, but the turtles were not blown ashore. Frequency of reports of cold-stunning in our study were also likely related to a higher likelihood that sea turtles which stranded in south-west WA (higher latitudes; colder waters) would be discovered and admitted to Perth Zoo (the primary hospital admitting sea turtles in WA); given that this region of WA is the most densely populated (with people) and is also relatively close to Perth Zoo.

4.4.2 Morbidity and mortality investigations

Generally, the diseases reported in our study were similar to those reported in other studies in WA and across Australia. The main differences included the lower frequency of different bacterial diseases reported in our study compared to those reported by Raidal et al. (1998). One potential reason for this may be the infrequent use of microbiology in our study, which was related to the unsuitability of most of the cadavers in our study for microbiological research; in our study, 54/75 (72.0%) cadavers were frozen and/or autolysed, while the Raidal et al. (1998) study used only fresh specimens. Gordon (2005) also reported similar results; specifically, observing that bacterial diseases were uncommon, and usually only secondary to trauma or parasites. With regards to mycobacteria, similar to other Australian studies, our study found an absence of mycobacteria (Raidal et al. 1998, Gordon 2005). However, another Australian study (Flint et al. 2010d), did report mycobacterial infections. An absence of mycobacterial infection in our study may also be related to the low level of specific testing undertaken.

Through morbidity and mortality investigations, our study detected diseases in sea turtles which had not been previously reported in Australian studies. Salt gland adenitis and microsporidial myopathy, which were both reported in our study, had not been reported in any previous Australian studies; but both these diseases have been previously reported overseas (Oros et al. 2011, Martinson et al. 2018), including

reports of microsporidial myopathy cases in New Zealand (Dr L. Tiller, Auckland Zoo, personal communication, 3 August 2017). Two other diseases observed in our study, gout and pseudogout, have also not been reported in previous Australian studies, but gout has been reported once in the Canary Islands (Orós et al. 2005). We note also the possibility that our case was a combination of gout and pseudogout; which has been reported to co-occur in reptiles (Jones and Fitzgerald 2009). Gout is usually associated with renal disease, which could occur for a number of reasons; including dehydration (Oros 2019a). Pseudogout, which affects the joints, can also be caused by chronic renal disease; but could also be triggered by joint damage – which in the sea turtle case in our study, may have been related to articular gout (Oros 2019b). Although renal oxalosis – a different depositional disease which presents with crystals in the renal tissues – has been reported in Australia and elsewhere (Gordon 2005, Stacy et al. 2008, Flint et al. 2010d), testing ruled out renal oxalosis in our study.

Another disease that was reported more frequently in our study, compared to other studies in Australia, was pneumonia. Most turtles in our study for which pneumonia was identified as the primary or contributory cause of mortality were found in the southern parts of the state, and this was potentially associated with cold-related immunosuppression, given the tendency for cold-stunned cases to present with respiratory, GI and systemic pathology. Not all pulmonary lesions detected in this study were considered pneumonia. Many turtles had focal pulmonary lesions on necropsy; which were not considered contributory to mortality at the time, but had the potential to develop into significant life-threatening pulmonary disease over time, given that turtles cannot cough (Boylan et al. 2017b).

With respect to our findings regarding GI endoparasitoses, while most GI endoparasitoses in our study were mixed infections (*Anisakis* sp., tetraphyllid and other larval cestodes), not all contributed to the morbidity and mortality of sea turtles in this study. These infections are not reported to cause significant pathology and mortality (for example, tetraphyllid larvae in the GI tract in cold-stunned Kemp's ridleys were reported to lack associated pathology (Innis et al. 2009). Even heavy

burdens of trypanorhynch larvae are not considered contributory to mortality (Stacy et al. 2017c). However, considering the extensive associated pathology in these post-hatchling loggerhead turtles in our study, tetraphyllid and trypanorhynch cestodiasis are considered highly likely to play some role in morbidity. Migrating parasites, while generally of low pathogenicity, can also play a role in the development of coelomitis (Glazebrook and Campbell 1990a). One such example of a migrating parasite is the nematode larvae of *Anisakis* sp., which has the marine mammal as the definitive host and sea turtles as paratenic dead end hosts (Santoro et al. 2010).

In our study, GI impaction and ileus were reported in low numbers relative to other studies (Gordon 2005, Flint et al. 2010d). This may be related to other studies having access to fresh specimens, which facilitated the observation of subtle signs such as congestion, oedema and mucosal pathology. However, these findings were unable to be observed in the frozen and autolysed specimens available in our study given the propensity for the GI tract to autolyse.

My study's findings regarding FP and mortality showed differences and similarities to those reported in other disease and mortality studies in Australia. In our WA study, only 1.3% of total mortality cases were considered to have FP as a contributor to mortality, compared with 7% (Gordon 2005). However, my study's findings were comparable to 0.7% mortality observed by Flint et al. (2010d).

Although coccidiosis has been reported in parts of Australia (Gordon et al. 1993, Flint et al. 2010d, Chapman et al. 2016a) as well as in WA (Reinhold 2015), after discussions with the author of the WA study (L. Reinhold, personal communication, 22 January 2022) it was confirmed that no parasitological examinations were performed. Further examination of photographs provided by the author led to the conclusion that the parasites described as coccidia in the paper were most likely trypanorhynch cysts. Consequently, coccidia have not been reported previously in WA, and in our study, we found no evidence of coccidiosis (although coccidia was reported in two faecal floatation tests in our study). We were unable to determine whether the lack of coccidiosis observed in sea turtle mortalities in WA is related to autolysis, in particular

of the GI tract (as mentioned previously), or to subclinical disease, or because this disease is endemic and did not have the same impact with respect to mortalities as when the disease disseminated across the Pacific Ocean and emerged in the US (Stacy et al. 2019a). However, as coccidiosis is considered a significant disease in other sea turtle populations, it is recommended that surveillance to detect coccidiosis in WA sea turtles is continued.

Spirorchiidiasis

While spirorchiidiasis was clearly the most commonly observed primary and contributory cause of mortality for sea turtles in our study, it is also a particularly conspicuous disease and is easily diagnosed both grossly and histologically; so detection rates may be slightly higher than for other diseases, especially for those with subtle pathology. While it may be easier to detect adult spirorchiids in fresh sea turtle cadavers through observations of movements (Flint et al. 2009b), eggs remain readily identifiable with freezing and autolysis. In this study, sea turtles in the northern region were observed to have a higher prevalence of spirorchiidiasis ($p=0.034$) and more 'high' grade spirorchiid infections ($p=0.001$), which was likely related to the distribution of the intermediate host (such as marine gastropods and polychaete worms), with higher densities of these intermediate hosts in warmer, northern tropical climates. Similarly, Flint et al. (2010d) reported high levels of spirorchiidiasis and severe spirorchiidiasis (GI) in the warmer seasons compared to the cooler seasons in the Moreton Bay region, which was despite the temperate climate of this region. This seasonal difference was most likely related to cercarial emergence and higher densities of intermediate hosts during the warmer seasons. As expected, no oceanic or post-hatchling turtles had spirorchiidiasis, including a post-hatchling flatback (the only species without an oceanic life-stage) which had organ washes performed due to the risk of post-hatchling flatbacks being exposed to intermediate hosts in the nearshore environment.

Clinical investigations

When examining clinical signs for spirorchiidiasis and treatment for spirorchiidiasis of live turtle cases in this study, and comparing results from these clinical signs and treatment, it becomes clear that these clinical cases require more consistent record keeping, additional clinical assessment and further research. Turtles with spirorchiidiasis had a range of non-specific clinical signs, including cachexia, lethargy, and a quiet mentation. Although Gordon et al. (1998a) reported that oedema was a commonly-observed non-specific clinical sign, the majority of the cadavers in our study were frozen, so it was unclear if the coelomic fluid was artefactually increased in these cases. In our study, for turtles with neurospirorchiidiasis, neurological signs were not always reported, and a neurological examination was not routinely performed to detect abnormalities. However, as neurological spirorchiidiasis is such an important disease of sea turtles, it is recommended to incorporate a neurological examination in future diagnostic disease investigations (Chrisman et al. 1997, Jacobson 2006). Although adult parasites were still detected in some turtles treated with praziquantel, the reporting of treatment at rehabilitation centres in the regions was incomplete. Therefore, it cannot be ascertained whether the correct or complete course of praziquantel was provided. It is also possible that the dose rate for cestodiasis (8 mg/kg two weeks apart) is too low for spirorchiidiasis; some researchers have suggested that an appropriate treatment regime may require 25 mg/kg (or possibly higher) (Adnyana et al. 1997a, Jacobson 2003), given orally for three days (Dr D. Mader, Marathon Vet Hospital, Florida, personal communication, 11 October 2017). The efficacy of this treatment regime against adult spirorchiids could be assessed during future studies, if accurate treatment records are kept.

Pathology

While many of the spirorchiid-associated pathologies reported in our study had been reported previously in other Australian studies, our study reported few cases of bacteria associated with spirorchiids, when compared to reports from other studies. In our study, spirorchiid granuloma presentation was similar to the presentation

described in other studies; predominantly with histiocytic inflammation. Occasionally a heterophilic response was detected; which in other studies was typically attributed to bacteria detected through microbiological investigation (Gordon 2005). However, migrating spirorchiid eggs are also reported to spread bacteria (Gordon et al. 1998a). While we occasionally found bacteria associated with spirorchiids, we may have missed some of these bacteria-associated spirorchiid cases; as microbiology was only performed infrequently (Raidal et al. 1998). However, even in fresh cases, the lack of detection of bacteria using microbiology is a common problem (Gordon 2005). Similar to other Australian studies on green turtles, aneurysm was a common finding in green turtles in our spirorchiid study group (Gordon 2005, Flint et al. 2009b); in contrast to findings from some overseas studies, where aneurysm is reported to occur more rarely (Stacy et al. 2010a). Our study also supported the Flint et al. (2010d) study in finding that most of the spirorchiid-associated lesions in the brain were mild to moderate, while lesions within the meninges were more frequently severe.

All spirorchiid species and genera reported in this study have been previously reported in other WA and Australian studies, except in flatback turtles (Glazebrook and Campbell 1990b, Cribb and Gordon 1998, Platt and Blair 1998, Raidal et al. 1998, Chapman et al. 2015, Flint et al. 2015b). However, some of the spirorchiid genera reported by Raidal et al. (1998) for WA sea turtles were not observed in our study. These genera are most frequently observed in the mesenteric arteries and veins, which were not examined in detail in our study. To find these genera in future studies, we recommend investigating these vessels thoroughly. The only spirorchiid (species) detected during this study which has not been previously reported in flatback turtles is *Neospirorchis* sp. (Chapman et al. 2019). No adult spirorchiids have been reported in olive ridley turtles previously (only spirorchiid eggs). However, in our study, adult spirorchiids were seen in the brain histopathology of an olive ridley turtle which was subsequently diagnosed with neurospirorchiidiasis as the cause of mortality. The presence of spirorchiidiasis in the flatback turtle in this study was not an isolated case. Another flatback turtle from the NT (not included in this study) and necropsied as part of the 2016 Australian Sea Turtle Symposium, had histopathology performed by the

author at Murdoch University and mild spirorchiidiasis was detected. A potential reason why flatback turtle strandings are rarely observed by humans include the species' restricted distribution and their habitation of remote areas which are difficult to access. Another potential hypothesis is that the flatback turtle is at higher risk of predator attack as this species inhabits areas with relatively high numbers of predators (their life-cycle is entirely on the continental shelf) (Bolten 2003), and cases of acute fatal trauma including predation are less likely to be recovered. Our study found adult spirorchiids in sea turtle cadavers through organ washes in body systems where adult spirorchiids are not normally detected (i.e., in the respiratory, renal and even haematopoietic systems). We detected *H. mistroides* in a green turtle which according to Chapman et al. (2019), this species has only been previously reported in hawksbill and loggerhead turtles. Our study also found a *Neospirorchis* sp. adult, which is also an uncommon finding, highlighting the importance of organ washes (Chapman et al. 2019).

An adult spirorchiid, identified as *Carettacola* sp., was also found in the organ wash of a liver of one of the two healthy flatback turtles (WT82) which died from misadventure (cliff fall) during nesting at Mundabullangana station in the Pilbara (Figure 4.6). This occurred outside the study period, and the results were therefore not included in the analysis of morbidity and mortality cases in this chapter (E. Young unpublished data).

Affected body systems, detection methods and risk factors

We found that while most body systems were statistically correlated with other body systems for presence of spirorchiidiasis in stranded sea turtle cadaver cases, fewer body systems were significantly correlated for severity of spirorchiidiasis. Our findings that the incidence and severity of spirorchiidiasis in the respiratory system, cardiovascular system, digestive system and haematopoietic system were correlated, was similar to the findings of Flint et al. (2010d). However, Flint et al. (2010d) also found that none of these body systems correlated with the neurological system, but we found correlation between the incidence of spirorchiidiasis in the neurological system and each of these body systems, and between the severity of spirorchiidiasis

in the neurological system and each of these body systems, except the cardiovascular system was not correlated. The correlation of spirorchiidiasis in certain body systems is expected owing to their important role in the pathophysiology of disease (i.e., the digestive and respiratory system act as potential entrance and exit points, and the spleen serves as a highly vascularised filtration organ where eggs may accumulate) (Work et al. 2005, Anderson et al. 2017, Chapman et al. 2019). The lack of correlation of spirorchiidiasis in the cardiovascular and neurological systems may be related to the predilection of different spirorchiid species for site specific locations within the host (Flint et al. 2010d).

Adult spirorchiids were most commonly found in the cardiovascular system, neurological system, haematopoietic system and respiratory system. Few spirorchiid flukes were found in the digestive system; probably because organ washes were not performed on the digestive system, due to the difficulty of retrieving spirorchiids from this system using standard organ wash techniques. Most disease studies where spirorchiids were collected at necropsy (with or without organ washes), examined the heart and great vessels; and consequently, in those studies, the majority of spirorchiids were found in the major cardiovascular system (Gordon 2005, Flint et al. 2015b). In those same studies, with the use of microscopic techniques, spirorchiids were also found in other systems, including the neurological system and the GI system (and occasionally the respiratory system), but spirorchiids were absent from the haematopoietic system. In studies with more detailed parasitological examination, spirorchiids were also found in the liver and intestinal vasculature (Platt and Blair 1998, Raidal et al. 1998) (A. Elliot, Murdoch University, personal communication, 22 September 2016).

Although organ washes in our study seemed to produce a low rate of spirorchiid detection, the spirorchiid detection rate (19/148) appeared notably higher than the detection rate using gross necropsy (2/148), and appeared slightly higher than when using histopathology (11/148). Although organ washes are time-consuming, the potential for organ washes to enable the detection of more spirorchiids than necropsy

and histopathology, highlights the potential value of organ washes in conjunction with other diagnostics in future studies investigating spirorchiidiasis.

Our study did not detect a significant correlation between age-class category of sea turtle and occurrence of spirorchiidiasis (p=1) or between age-class and severity of spirorchiid infection (p=1), possibly related to the small sample size for adults (n=8). In contrast, Flint et al. (2010d) found spirorchiids were significantly more likely to cause severe pathology in immature green turtles than in mature green turtles. Similarly, Raidal et al. (1998) hypothesised that high frequency of observations of juvenile green turtle strandings, compared with other age-classes may be due to an increased susceptibility of juveniles to spirorchiidiasis. It is generally thought green turtles are particularly susceptible during their ontogenetic shift when they transition from the open ocean to the near shore environment, simultaneously changing from an herbivorous to carnivorous diet. In contrast to Flint et al. (2010d), Stacy et al. (2010a) found the occurrence of spirorchiids and severity of spirorchiid infection were significantly higher in mature turtles than in immature turtles. However, our study did find a significant correlation between BCS and spirorchiidiasis of the haematopoietic system, a finding similar to Work et al. (2005) which found splenic egg intensity was inversely related to BCI. This correlation is possibly related to the organ's role in filtration where splenic accumulation of eggs may reach a threshold of parasite intensity, above which disease ensues and body condition is impacted. It remains challenging to evaluate the significance of spirorchiidiasis on sea turtle health status overall, as the presence of spirorchiid eggs and adults, and the intensity of spirorchiid infection, are not always correlated with a pathological response. Therefore the impact on turtle health can be difficult to assess (Gordon 2005). It is therefore imperative that diagnosis of spirorchiidiasis as a cause of mortality is made with caution, taking all results from a variety of diagnostics into consideration (Stacy 2008).

The Australian Sea Turtle Recovery Plan (Commonwealth of Australia 2017b) currently lists spirorchiidiasis as a low (unknown) threat; however, diseases like spirorchiidiasis may have more impact on populations than previously estimated. Spirorchiidiasis has

been referred to as a disease of 'intermediate pathogenicity' (Gordon 2005). Such diseases can have more impact on a population than a pathogen that causes immediate death (Gordon 2005). Further, considering the incidence of spirorchiidiasis and severity of disease was significantly higher in warmer, northern regions than the cooler, southern regions (most likely related to the presence of the intermediate host) (Flint et al. 2010d), spirorchiidiasis poses a significant threat to sea turtle populations at lower latitudes, further highlighting the critical nature of understanding this disease.

Marine debris

No published studies in WA have reported marine debris ingestion in sea turtles. This includes a necropsy study by Reinhold (2015) targeting marine debris in the GI tract in 20 turtles (2002-2013), and mortality studies involving green and loggerhead turtles from the mid-north coast of WA (1997 and 2003 respectively) (Dr R. Prince, DBCA, personal communication, 20 August 2022) (Raidal et al. 1998, Raidal et al. 2006). In contrast, we found marine debris in 12.0% of the WA stranded sea turtle cases. In two juvenile green turtles, marine debris was the definitive cause of mortality in one case (WT29), and in another green turtle case (WT71), marine debris was reported to be associated with a colonic obstruction and considered to have contributed to mortality. Most of the turtles which had ingested marine debris were post-hatchlings (66.7%), specifically, both loggerhead and flatback turtles. The most common item found was pieces of synthetic line, most likely from discarded fishing nets (Duncan et al. 2021). Post-hatchlings and small juvenile turtles, which are indiscriminate and surface feeders, are the most at risk age-class with respect to marine debris ingestion (Milton and Lutz 2003).

Although marine debris did not appear to be the cause of or contribute to mortality in the majority of stranded sea turtle cases, this does not mean there are not subclinical effects from marine debris, such as dilution of nutrients, ingestion of toxic plasticising compounds, or biotoxins adhered to the debris (McCauley and Bjorndal 1999, Thevenon 2014). At the same time, many studies attribute the presence of marine debris within the GI tract to be the cause of mortality. However, without the use of

histopathology it may be difficult to determine whether gross observations represent actual pathology or post-mortem artefact. It is clear that the problem of marine debris in our environment is immense, and the study of marine debris ingestion by sea turtles can help to indicate the density of marine debris in sea turtle habitat. However, it is also important to ensure accurate reporting of the pathology associated with marine debris (Lynch et al. 2019). While it is possible that marine debris may cause perforations and other associated pathology, Lynch et al. (2019) argue that Wilcox et al. (2018) may have misinterpreted apparent pathology of the GI tract in autolysed cadavers. To increase accuracy of a diagnosis of marine debris associated pathology, histopathology should be included in marine debris studies, to confirm presence of histopathological lesions associated with marine debris.

Trauma

Trauma is another category for which disease investigations should accompany stranding surveys. In our study, before disease investigations were undertaken, the trauma stranding category was incorrectly attributed initially in 5/17 (29.4%) cases (with trauma-related cause of death such as bacterial nephritis due to tail injury included in the correctly attributed count). Similarly, Gordon (2005) found that when cause of death was not investigated in stranding surveys by the Queensland National Parks and Wildlife Service, vessel strike was overestimated by 40%. In our study, vessel strike was identified as the cause of mortality in 3/75 (4.0%) of cases, which is a minimum only due to trauma cases of unknown cause (2/75, 2.6%), whereas vessel strike contributed to mortality in a minimum of 4/75 (5.3%) cases.

In our study, all turtles identified as healthy and in good body condition prior to death (n=3) were trauma cases. This finding supports those reported by Barco et al. (2016), who found turtles that died acutely (from vessel strike or fishery interaction) were healthy prior to the traumatic event. Another study in Florida found that some turtles presenting with trauma had pre-existing conditions (Foley et al. 2019). Vessel strike is the health threat of sea turtles that is most likely to result in death, even if turtles survive the initial incident, death may occur from ongoing injury-related impacts; as is

indicated by the finding that turtles with vessel strike injuries have been found to be in a lower rated body condition than turtles without vessel strike injuries (Foley et al. 2019).

When reviewing the types of diseases identified in stranded sea turtles by rehabilitation status, we noted the following observations. Trauma was less likely to occur in turtles being rehabilitated than non-rehabilitated turtles, while cold-related immune altering diseases such as pneumonia and endoparasitoses were more likely to occur in rehabilitated turtles than in non-rehabilitated turtles. This is probably because trauma cases are more likely to be found dead or require euthanasia on arrival, whereas non-trauma cases are more likely to have chronic diseases, and hence more likely to be found alive and taken into captivity for rehabilitation. Furthermore, many turtles stranding in the South-West were cold-stunned post-hatchlings affected by pneumonia and endoparasites, and were more likely to enter rehabilitation due to the proximity of the stranding location to Perth Zoo.

To improve accuracy of a diagnosis of trauma, histopathology can be utilised to determine whether trauma occurred ante-mortem. Turtles with obvious external injuries and which are otherwise in good condition should still be necropsied, as these turtles can still die from other reasons and are an underrepresented cohort of stranded sea turtles in our study (as are healthy turtles which die acutely). Investigating these turtles can contribute to knowledge about sea turtle diseases and normal background pathology in healthy turtles.

4.4.3 Associations between ante-mortem and pathological findings

Although ante-mortem results were useful to have, to compare with post-mortem findings, in general, we did not observe any associations between post-mortem results and clinical signs, clinical pathology, or treatment. Our findings support those of Flint et al. (2010d), who found no correlation between clinical signs and pathological findings, except for neurological signs and neuropathology. In our study, there were a few exceptions to this general finding. For example, while uric acid is reported to be

the best indicator for renal disease (Stacy and Innis 2017), in our study, stranded turtles with values three times more than upper limit for uric acid had normal kidneys. However, turtles with uric acid nine to 14 times higher than the upper limit did have interstitial nephritis and tubular necrosis. Another example of the assessment of associations between ante-mortem and post-mortem results is the lack of correlation between CK and myopathy. In our study, the turtles with the highest CK values all had GI pathology; such as endoparasitoses and ulceration from marine debris ingestion. These findings of high CK in turtles with GI pathology support earlier research which has reported a wide distribution of enzymes and high levels of CK in muscle, heart and in the GI tract (Anderson et al. 2013). However, the Anderson et al. (2013) study was conducted in loggerheads, and different species have been shown to have different distribution of enzymes (Petrosky et al. 2015), highlighting the current rudimentary understanding of biochemical analytes in reptiles.

4.4.4 Limitations and recommendations

Major limitations for this project were the logistical and financial challenges to accessing fresh cadavers; associated with the geographical extent of WA's coastline. Access to fresh cadavers has likewise been identified as a challenge for other studies, in both WA (Raidal et al. 1998) and elsewhere (Stacy et al. 2010a). Cadaver decomposition is a particular problem for researchers relying on turtle cadavers from northern parts of Australia and from remote areas which are infrequently patrolled/visited (Cook et al. 2020). While 73.3% of cadavers in this study were either D1 or D2 (including frozen cadavers), only 28.0% of the total cadavers were necropsied while fresh and in good condition (i.e., D1 or D2; excluding frozen cadavers), with the remaining 4.0% of the fresh cadavers in poor condition. Frozen turtles accounted for 68.0% of all cases in our study (including D2-D4 cadavers). However, if trained personnel had been available, or if the turtle cadaver had been transported to Perth, then 73.3% of stranded turtle cases could have been necropsied fresh and in good condition (i.e., D1 or D2). In similar studies (Raidal et al. 1998, Gordon 2005, Flint et al. 2010d), researchers had access to all/mostly fresh cadavers. The Raidal et al. (1998)

study was specifically investigating an increase in stranding numbers in WA; and as such, fresh cadavers were made available. The Queensland studies were located over a smaller area (i.e., south-east Queensland covers approximately 400 km of coastline, compared to 21,000 km for the WA coastline in our study). In the Queensland studies, stranded turtle cadavers were also more frequently found in fresher condition than in our study, as a higher proportion of the Queensland coastline is well-populated compared to our study; and with shorter distance to laboratories.

When comparing the cadaver reporting and suitability results from our study with those of previous studies, less turtle strandings were reported in the state of WA (n=191 stranded turtles reported over the three year study; average of 63 turtles reported per year, WAStD accessed 21 April 2021) than in south-east Queensland (100-255 turtles are reported each year). Despite the challenges with accessing and reporting of cadavers in WA, numbers of cadavers suitable for necropsy in WA were comparable with numbers of suitable cadavers in Queensland. In Queensland 10-33% were suitable for necropsy (Gordon 2005, Flint et al. 2010d), whereas in our study 75/191 (39.3%) of the total cases stranding in WA were necropsied. However, only 56/191 (29.3%) of the total stranding cases were suitable for pathology (i.e., D1 or D2), and only 21/191 (11.0%) of these cases were necropsied fresh.

As a large state, WA needs to continue to develop a coordinated stranded turtle reporting network, with funding directed to these projects to enable them to run effectively. Until this occurs, the absence of a coordinated reporting network will remain a barrier to obtaining fresh cadavers and adequate sample sizes.

Given the logistical and financial constraints of transporting fresh sea turtle cadavers up to 3000 km from remote areas to Perth for diagnostic testing, freezers had already been placed in many regional DBCA offices. Cadavers will continue to need to be frozen until DBCA staff, veterinarians, biologists, wildlife rehabilitators and other affiliated personnel in regional areas have the skills and capacity to perform necropsies in these regional locations and collect samples. Once regional areas become independent and build capacity, photographic and other documents plus samples can be sent to

researchers in Perth, which will reduce the reliance on researchers travelling to regional areas to perform necropsies, and reduce the financial costs associated with transporting large, heavy animals.

The challenge associated with cadavers and samples being frozen in this study had implications for detecting disease through histopathology (post-mortem artefact, especially in autolysed samples, precluding diagnoses and subtle pathology detection); microbiology (samples were generally unsuitable, with contaminant overgrowth or bacteria killed by freezing); parasitology (parasites were less likely to be detected through movement, and are difficult to identify in decomposed/frozen samples). As expected, our study found that a definitive cause of death was able to be made more frequently in cadavers that were in good condition (D1 and D2, including frozen cadavers; 54/56, 96.4%) compared with decomposed cadavers (9/19, 47.4%) ($p<0.001$).

Although all sea turtle species that are present in WA, except the leatherback, were examined in this study, for some species and age-classes the sample sizes were limited, which in turn limited the capacity for statistical testing. We recommend that further research should investigate under-reported boundary groups to fill remaining knowledge gaps with respect to species, age-classes, regions (Mid-West, Northern Kimberley) and heavily industrialised areas. Further investigations into sea turtle disease as well as research into normal background pathology, should also be undertaken to improve our understanding in these fields, for example by investigating potential anthropogenic factors of acute death cases in otherwise healthy turtles. Health surveillance programs should also be incorporated into any ongoing or upstarting monitoring activities.

In this study, stranded turtle case selection was opportunistic; and biased towards chronic cases, given the greater likelihood of finding chronically debilitated animals than animals with disease of acute duration. These cases with chronic disease often had multi-factorial disease, which complicated making a definitive diagnosis. Although most trauma cases in our study did not appear to have underlying disease (6/10), this

is not generally the case with wildlife mortality studies (Stacy et al. 2017b, Stacy et al. 2017c). Potential reasons for a lack of underlying disease in trauma cases could be related to cadaver and sample quality as many of our study samples were affected by autolysis and freezing. Subtle pathology is more likely to be missed with poor sample quality, which may in turn, preclude the diagnosis of underlying disease. The sample size of trauma cases was also reduced because three potential trauma cases were removed from the analysis due to decomposition.

As mentioned, when turtles had multiple diseases present, we determined the most likely cause of mortality by using the available evidence; and this necessarily involved some subjectivity. In particular, spirorchiidiasis is a challenging disease to assign as a cause of or contributor to mortality, given that turtles with severe pathology associated with spirorchiidiasis often appear to be in good body condition at necropsy. Even apparently 'healthy' nesting turtles killed by jaguars in Costa Rica have been found with severe spirorchiidiasis at necropsy (Santoro et al. 2007). In WA, healthy nesting flatback turtles (WT82 and WT83) necropsied outside of the study which died of misadventure (cliff fall), had evidence of spirorchiidiasis including the presence of an adult spirorchiid in WT82. For this disease, the exact point at which pathology starts to affect health is unknown.

Another limitation of this study was the inconsistency of stranding records. Some records reported the presence of abnormalities, but it was unclear whether a lack of information about abnormalities in a record meant that abnormalities were absent, or were simply not recorded. For example, buoyancy disorders were reported for 27/45 (60.0%) live turtles. However, it is possible that the result of 23/27 (85.2%) cases with buoyancy disorders is skewed, as floating turtles are more likely to be reported than turtles with normal buoyancy. Although there is a high likelihood that stranded turtles will float, it is recommended that the presence or absence of floating should be recorded in future records. This will help researchers to determine the frequencies and proportions of turtles with buoyancy disorders. A similar limitation occurred for the clinical examination of live turtles, where there was an inconsistency in reporting

because examination was undertaken by the attending veterinarian (not a single investigator i.e., the author), and the turtle was treated as a clinical case and not as a research case. Additional recommendations regarding veterinary care, diagnostics, treatment and record keeping are found in Chapter six – General discussion.

Another limitation of this study was that for the stranded turtle cases, we did not rule out other differential diagnoses (such as toxicities), for example in neurospirorchiidiasis cases. Many questions remain to be answered by further research, including the impacts of indirect anthropogenic threats on sea turtles in WA.

4.4.5 Conclusions

This project has developed a baseline of diseases and causes of mortality of sea turtles in WA. It has highlighted important research gaps for which more research is required. This study has contributed to state-, national- and international-level knowledge about diseases of sea turtles in WA and the east Indian Ocean. It has added to the existing global knowledge of sea turtle diseases, including through the identification of novel diseases, range extensions, and potentially emerging diseases of importance to sea turtles.

While causes of sea turtle strandings globally remain largely unknown, this project has reported the importance of utilising disease investigations to help to determine cause of death. Although disease investigations may not always enable researchers to reach a diagnosis regarding cause of death, thorough disease investigations are likely to increase the frequency and accuracy of diagnosis of causes of morbidity and mortality.

This study supports other Australian studies of sea turtle mortalities in finding a relatively high frequency of natural causes of death rather than direct anthropogenic causes of death. However, our findings also highlight the impact of disease on sea turtles in this state; given our findings that natural disease was a cause of mortality in the majority of cases (69.3%). Research on green turtles in Queensland has likewise shown that disease features more prominently in mortality reports than in stranding surveys (Gordon 2005). Given these observations, we recommend that necropsies

continue for all sea turtle cadavers, and that access to fresh cadavers becomes a high priority. This would be particularly valuable for understudied and endangered species and age-groups, including hawksbill, olive ridley, flatback, leatherback (and loggerhead, excluding post-hatchlings). This study represents a platform from which to continue to develop a disease and mortality baseline. From here, we can begin to monitor sea turtle populations more thoroughly and systematically, with the aim of ensuring their health and persistence. Only by better understanding the causes of death for sea turtles can we understand the threats to their health, and manage these threats effectively to ensure the long-term survival of sea turtle species in Australia and globally.

CHAPTER 5

Streptococcus iniae associated mass marine fish kill and first report in sea turtles and sea snakes off Western Australia

This chapter is a modified version of the following paper:

Young, E. J., J. Bannister, N. B. Buller, R. J. Vaughan-Higgins, N. S. Stephens, S. D. Whiting, L. Yeap, T. L. Miller, and K. S. Warren. 2020. " *Streptococcus iniae* associated mass marine fish kill off Western Australia." Diseases of Aquatic Organisms 142: 197-201.

Modifications of the original published manuscript were made to suit the style and formatting of the thesis. Additional results for the sea turtles and sea snakes found during the mass mortality event are also included in this chapter (and in the supplementary material in Appendix 19).

Abstract

Streptococcus iniae causes high mortality in cultured and wild fish stocks globally. Since the first report in captive Amazon river dolphins (*Inia geoffrensis*) in 1976, this pathogen has emerged in finfish across all continents except Antarctica. In March 2016, dead and dying marine life including an estimated 17,000 fish were observed along a remote 70 km stretch of the Kimberley coastline north of Broome, WA. Affected species included finfish (lionfish [*Pterois volitans*], angelfish [*Pomacanthus* sp.], stripey snapper [*Lutjanus carponotatus*], sand bass [*Psammoperca waigiensis*], yellowtail grunter [*Amniataba caudavittata*], damselfish [*Pomacentridae* sp.]), flatback sea turtles (*Natator depressus*), and olive (*Aipysurus laevis*) and black-ringed (*Hydrelaps darwiniensis*) sea snakes. Moribund fish collected during the event exhibited exophthalmia and abnormal behaviour, such as spiralling on the surface or within the water column. Subsequent histopathological examination of two fish species revealed bacterial septicaemia with chains of Gram-positive cocci seen in multiple organs and within brain tissue. In fish and sea turtle samples, *S. iniae* was identified by bacterial culture, species-specific PCR, Matrix-Assisted Laser Desorption Ionisation Time-Of-Flight (MALDI-TOF) and biochemical testing; and in sea snake samples, *S. iniae* was identified by species-specific PCR. DNA fingerprinting using infrequent restriction site PCR (IRS-PCR) on isolates from fish and sea turtle samples indicated the fish and sea turtle strains were identical. This is the first report of *S. iniae* associated with a major multi-species wild marine fish kill in Australia, and the first detection of *S. iniae* in threatened sea turtle and sea snake species. Extreme weather

events in the region including a marked decrease in water temperatures, followed by an extended period of above-average coastal water temperatures, were implicated as stressors potentially contributing to this outbreak.

5.1 Introduction

Streptococcus iniae is an important aquatic bacterial pathogen responsible for significant disease outbreaks in a diverse range of finfish species in both freshwater and marine environments globally (Agnew and Barnes 2007). Disease is primarily reported in aquaculture, but *S. iniae* has also been implicated in several wild fish kills (Foo et al. 1985, Perera et al. 1994, Eldar et al. 1995, Zlotkin et al. 1998, Ferguson et al. 2000, Colorni et al. 2002, Keirstead et al. 2014). This agent has also been isolated from captive Amazon river dolphins (*Inia geoffrensis*) (Pier and Madin 1976), cultured American bullfrogs (*Lithobates catesbeianus*) (Mauel 2002) and a captive bottlenose dolphin (*Tursiops truncatus*) in China (Song et al. 2017). *Streptococcus iniae* is zoonotic and has been reported in humans in the USA, Canada, Hong Kong, Singapore and Taiwan (Weinstein et al. 1997, Facklam et al. 2005, Sun et al. 2007). In Australia, *S. iniae* was first isolated from sea-cultured barramundi in Queensland in 1999 (Bromage et al. 1999). While in WA, the disease first appeared in freshwater barramundi cage culture at Lake Argyle in 2003, causing substantive losses which resulted in closure of the farm (Creeper and Buller 2006). In early March 2016, large numbers of dead and moribund marine organisms along the coastline north of Broome, WA (centred around 17.488°S, 122.143°E) were reported to WA government agencies responsible for management of the State's Fish Kill Response Program which subsequently investigated the outbreak. Here we report data implicating *S. iniae* associated with the first major multi-species wild fish kill in Australia since records have been kept. We also present the first record of *S. iniae* in reptiles, including the flatback turtle (*Natator depressus*) and olive sea snake (*Aipysurus laevis*).

5.2 Materials and methods

5.2.1 Aerial and beach transect surveys

Aerial surveys along the coast north of Broome determined that the fish kill extended over 70 km of coastline (between 17.240°S, 122.199°E and 17.767°S, 122.200°E). Beach transects were conducted at eight sites (20 × 150 m transects per site) within the survey area, to quantify and identify fish species and collect samples.

5.2.2 Fish

Dead fish were collected from Crocodile Creek (17.266°S, 122.175°E) on 15 March 2016 and placed into 10% NBF or chilled or frozen for transport to the DPIRD diagnostic laboratory 2000 km away in Perth, WA. No live fish were observed. On gross examination at the laboratory, tissues were unsuitable for detailed histological examination due to severe decomposition. Gills, liver, visceral adipose tissue, kidney and skeletal muscle from four freshly dead fish (sand bass [*Psammoperca waigiensis*], stripey snapper [*Lutjanus carponotatus*], angelfish [*Pomacanthus* sp.] and lionfish [*Pterois volitans*]) were submitted to the ChemCentre, Bentley, WA, for heavy metal, hydrocarbon and organic pesticide residue testing. These tissues from the four fish were also submitted to Advanced Analytical Australia, Perth, WA, for algal toxin analyses, including paralytic shellfish poisoning (PSP), amnesic shellfish poisoning (ASP), diarrheic shellfish poisoning (DSP) and neurotoxic shellfish poisoning (NSP).

Despite the autolysis of these initial tissue samples, preliminary histopathological examination detected numerous Gram-positive chain-forming cocci in the gills and heart, and subsequently fresh fish were requested for bacterial culture. At a second sampling on 19 March 2016, several marine fish species (including lionfish, stripey snapper, yellowtail grunter [*Amniataba caudavittata*], damselfish [*Pomacentridae* sp.], sand bass and angelfish) were collected live in water from multiple sites including Crocodile Creek and additional locations within the fish kill area. The fish were euthanised via chemical overdose with AQUI-S® (AQUI-S New Zealand) following the

American Veterinary Medical Association guidelines (American Veterinary Medical Association 2020) and couriered to the DPIRD diagnostic laboratory as before. Brains and hearts from each of the seven fish were dissected using aseptic technique and submitted for bacterial culture. No histopathology was performed on these individuals.

At a third sampling on 22 March 2016, five moribund marine fish (including four stripey snapper and one hussar [*L. adetti*]) were caught at sea within the fish kill region. The fish were euthanised and examined grossly for external and internal lesions. Gill, heart, brain, kidney, spleen, liver, intestine, stomach, skin and skeletal muscle were sampled from the five fish. Tissues were fixed in 10% NBF.

Fish tissues fixed in 10% NBF were processed at DPIRD using a LOGOS (Milestone Medical) histological processor and embedded in paraffin wax, sectioned at 4 µm and stained with H&E and Gram stains then examined by light microscopy.

5.2.3 Sea turtles and sea snakes

During the Broome mortality event, dead marine reptiles (including 12 juvenile flatback turtles, 16 olive sea snakes, and two black-ringed sea snakes [*Hydrelaps darwiniensis*]) were collected from the beach within the survey area and frozen before being transported to Murdoch University, Murdoch, WA. All reptile cadavers were in an autolysed state and an unexpected freezer malfunction at the University caused further deterioration of the samples. Eleven flatbacks and six sea snakes were suitable for necropsy and sampling as the remaining cadavers were severely autolysed. Histopathology was performed on two flatbacks, two olive sea snakes and two black-ringed sea snakes at Murdoch University using a Tissue Tek VIP 5 Jr (Sakura) processor and standard methodology. In 2017, bacteriological testing was undertaken at DPIRD on frozen flatback and sea snake tissues stored up to 18 months, with additional testing in 2020 (more than four years after the event).

5.2.4 Bacterial culture and identification

Sections of heart and brain from seven fresh fish (including lionfish, stripey snapper, yellowtail grunter, damselfish, sand bass and angelfish) and various tissues (including liver, heart, kidney, spleen, brain) from nine flatback turtles and five sea snakes (including olive and black-ringed sea snakes) frozen at - 20°C for more than 12 months without cryoprotectant and thawed at 4°C, were flame-sterilised in 95% alcohol and macerated before inoculation onto 5% horse blood agar, MacConkey agar (Oxoid, Thermofisher Scientific) and marine salt agar (Buller 2014) (prepared at DPIRD media laboratories). Plates were placed into a sealed container and incubated at 24°C in a refrigerated incubator (Biocell 1000, Contherm Scientific), and examined daily over six days. Growth was recorded as light, moderate or heavy, according to the amount of growth on the quadrants of the plate. Suspect colonies of *S. iniae* were identified using MALDI-TOF (BioTyper, Bruker Daltonics) with the MBT Compass Library DB-6903, conventional biochemical tests, and the API 20 STREP (Biomerieux) (Barrow and Feltham 1993, Buller 2014). Conventional biochemical tests for ornithine decarboxylase (ODC), lysine decarboxylase (LDC), arginine dihydrolase (ADH), nitrate reduction, indole, citrate, urea, methyl red, Voges Proskauer, ortho-nitrophenyl-β-galactosidase (ONPG), hydrolysis of gelatin, DNA and aesculin, and fermentation of arabinose, glucose, inositol, lactose, maltose, mannitol, mannose, salicin, sorbitol, sucrose, trehalose and xylose was performed on one representative isolate (fish isolate AS-16-0864-#5). The biochemical set was incubated at 24°C for 48 hours. Lancefield groups A to G were tested on isolate AS-16-0864-#5 using the Prolex Strep Grouping Kit A-G (Pro-Lab Diagnostics). Three fish isolates (AS-16-0864-#1 and -#5, and AS-16-0889-#8) and one flatback turtle isolate (AS-17-2548-#9) were tested using the API 20 STREP, which was inoculated according to the manufacturer's instructions, incubated at 24°C and read at 24 hours.

5.2.5 PCR and amplicon sequencing

Species-specific PCR (Mata et al. 2004) was used to detect *S. iniae* in tissues from flatback turtles and sea snakes, and as an aid to identify *S. iniae* in two representative fish bacterial colonies (AS-16-0864-#5 and -#7). DNA was extracted using the QIAamp DNA mini tissue kit and the Gram-positive bacterial pellet protocol with the pre-digestion step for tissue on a QIAcube nucleic acid extraction robot (Qiagen), and 5 µl was used in a final 25 µl reaction. A 0.2 µM final concentration of the *S. iniae*-specific primers, LOX-1 and LOX-2 (Mata et al. 2004), were used in a Master Mix (Promega) and tested at an annealing temperature of 60°C over 35 cycles on a DNA Engine thermocycler (Bio-Rad). Isolate AS-04-0018-#1, a confirmed *S. iniae* isolate from the 2003 Lake Argyle farmed barramundi mortality event (Creeper and Buller 2006), was used as a positive control.

To determine the relatedness of the *S. iniae* isolated from the dead fish and flatback turtles during the Broome mass mortality event, one fish isolate (AS-16-0864-#5) and two turtle isolates (AS-17-2548-#9 and -#14) were compared using a DNA fingerprinting method, IRS-PCR (Mazurek et al. 1996) with modifications according to Buller et al. (2010). Adaptor sequence and preparation followed the original paper by Mazurek et al. (1996). AH adaptor = (AH1 5'-AGA ACT GAC CTC GAC TCG CAC G-3' + AH2 = 5'-TGC GAG T-3'), AX adaptor = (AX1 5'- PO_4-CTA GTA CTG GCA GAC TCT-3' + AX2 5'- GCC AGT A-3'). The primers used at 0.2 µM in the PCR reaction were AH1 (5'-AGA ACT GAC CTC GAC TCG CAC G-3') and PXT (5'- AGA GTC TGC CAG TAC TAG AT-3'). Thermocycling was carried out on a DNA engine thermocycler (Bio-Rad) using the conditions recommended by the manufacturer.

Amplicons were trimmed from five PCR positive gel samples (two tissue samples and three bacterial colonies, including one positive control) using sterile scalpel blades which included turtle, sea snake and fish samples, and were sent to AGRF for Sanger sequencing. Sequences returned from AGRF were assembled to create a consensus sequence and submitted for a BLAST search to confirm the target amplicon.

5.3 Results

5.3.1 Aerial and beach transect surveys

A total of 37 species of fish were recorded during the beach transect surveys with an estimated 17,000 individuals involved in the event over the 70 km stretch of coastline.

5.3.2 Fish

Moribund fish showed abnormal circling behaviour and exophthalmia. No further lesions were detected in the fish examined at necropsy. No significant levels (i.e., sufficient to cause acute mass ecosystem-wide mortalities) of heavy metal, hydrocarbons, organic pesticide residues or harmful algal toxins (i.e., ASP, DSP, NSP and PSP) (Department of Health 2011) were detected in the tissues of the four freshly dead fish.

In all five of the freshly fixed euthanised fish (*L. carponotatus* and *L. adetti*), pathology was consistent with chronic bacterial septicaemia (Figure 5.1 and Figure 5.2). Inflammatory changes ranged from mild to severe and included epicarditis, interstitial nephritis and branchitis. Four fish had mild to moderate myocarditis, two fish had moderate to severe encephalitis and three fish also showed mild enteritis and gastritis. Gram-stained sections revealed the presence of large numbers of Gram-positive cocci forming chains within inflammatory cell infiltrates, with colonies particularly numerous in the gill lamellae and epicardium. There were no other apparent histopathological findings to suggest the involvement of other infectious (e.g., parasitic) or non-infectious (e.g., hepatotoxic) contributing factors.

5.3.3 Sea turtles and sea snakes

No sea turtles or sea snakes had any gross or microscopic pathological abnormalities, however, this may have been due to autolysis. While Gram stains for the flatbacks were indeterminant, the sea snake stains showed mixed bacteria (likely post-mortem

contaminants), except for one olive sea snake with large pure colonies of Gram-positive cocci in the liver and kidney without associated pathology.

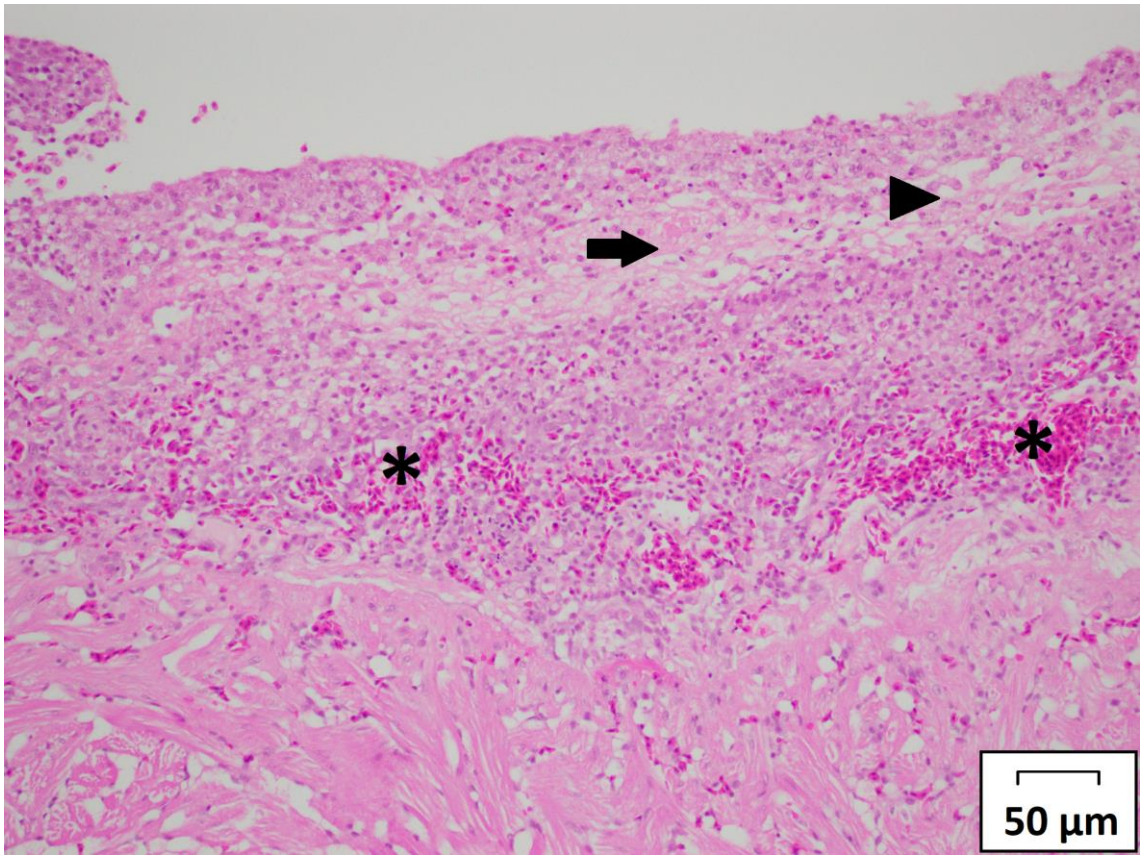


Figure 5.1 (H&E ×200). Myocardium and epicardium of the ventricle from a stripey snapper *L. carponotatus* showing severe diffuse chronic lymphocytic and histiocytic epicarditis with haemorrhage (asterisks), oedema (arrowhead) and fibrin (arrow) formation.

5.3.4 Bacteriological and molecular testing

A heavy to moderate growth of *S. iniae* (white, pinpoint, beta-haemolytic colonies at 24 hours) was obtained from brain and heart of lionfish, stripey snapper, yellowtail grunter, damselfish, sand bass and angelfish. *Streptococcus iniae* was isolated in three flatback turtles in a heavy to moderate growth from tissues including the kidney, liver, and heart, except for two colonies cultured from the spleen (see Table in Appendix 19 for more details). The MALDI-TOF scores for *S. iniae* were confident to species level. For the conventional biochemical tests, the fish isolate AS-16-0864-#5 was positive for hydrolysis of aesculin and negative for hydrolysis of gelatin and DNA, positive for fermentation of glucose, maltose, mannitol, mannose, salicin, sucrose and trehalose

and negative for fermentation of arabinose, inositol, lactose, sorbitol and xylose. The ODC, LDC, ADH, nitrate, indole, citrate, urease, methyl red and Voges Proskauer were negative. The ONPG was positive. These results closely matched the isolate reported in cultured barramundi from the Lake Argyle mortality event in 2003 (Creeper and Buller 2006). However, the barramundi isolate had a positive reaction to ADH after 72 hours incubation, whereas the Broome mortality event isolate was negative for ADH at 24 hours. As expected for *S. iniae*, the fish isolate AS-16-0864-#5 was negative for Lancefield groups A, B, C, D, E, F and G. The results for the API 20 STREP for isolates from three finfish and one flatback turtle were positive for aesculin hydrolysis, beta-glucuronidase, alkaline phosphatase, leucine amino peptidase, arginine dihydrolase, and positive for fermentation of ribose, mannitol, trehalose, starch and glycogen. Negative results were obtained for Voges Proskauer, hippurate, alpha-galactosidase, beta-galactosidase and fermentation was also negative for arabinose, sorbitol, lactose, inulin and raffinose. The isolates from the finfish were negative for pyrrolidonyl arylamidase, but the isolate from the turtle had a weak reaction and was difficult to interpret. The API 20 STREP bionumber for the finfish isolates was 4463117, and the turtle isolate was 4462117. The biochemical and API 20 STREP results were consistent with reports for *S. iniae* in the literature according to Buller (2014).

The species-specific PCR detected *S. iniae* in the fish bacterial isolates (AS-16-0864-#5 and -#7) including the positive control (AS-04-0018-#1), and in tissues from three flatback turtles with culture-positive results. An additional flatback turtle and an olive sea snake which were culture-negative, also received PCR-positive *S. iniae* results (flatback and sea snake results presented in Appendix 19). The isolate AS-16-0864-#5 from a fish and two turtle isolates (AS-17-2548-#9 and -#14) had the same DNA fingerprint using the IRS-PCR protocol. An amplicon at the correct size of 870 base pairs was produced in the species-specific PCR for the two tissue samples and three bacterial colonies, including the positive control. Amplicons from two tissue samples and two bacterial colonies were 99.3% to 99.8% identical to *S. iniae* accession number EU086704 across 657-671 nucleotides (nt), and one bacterial colony was 99.3% identical to *S. iniae* accession number CP032401 across 850 nt.

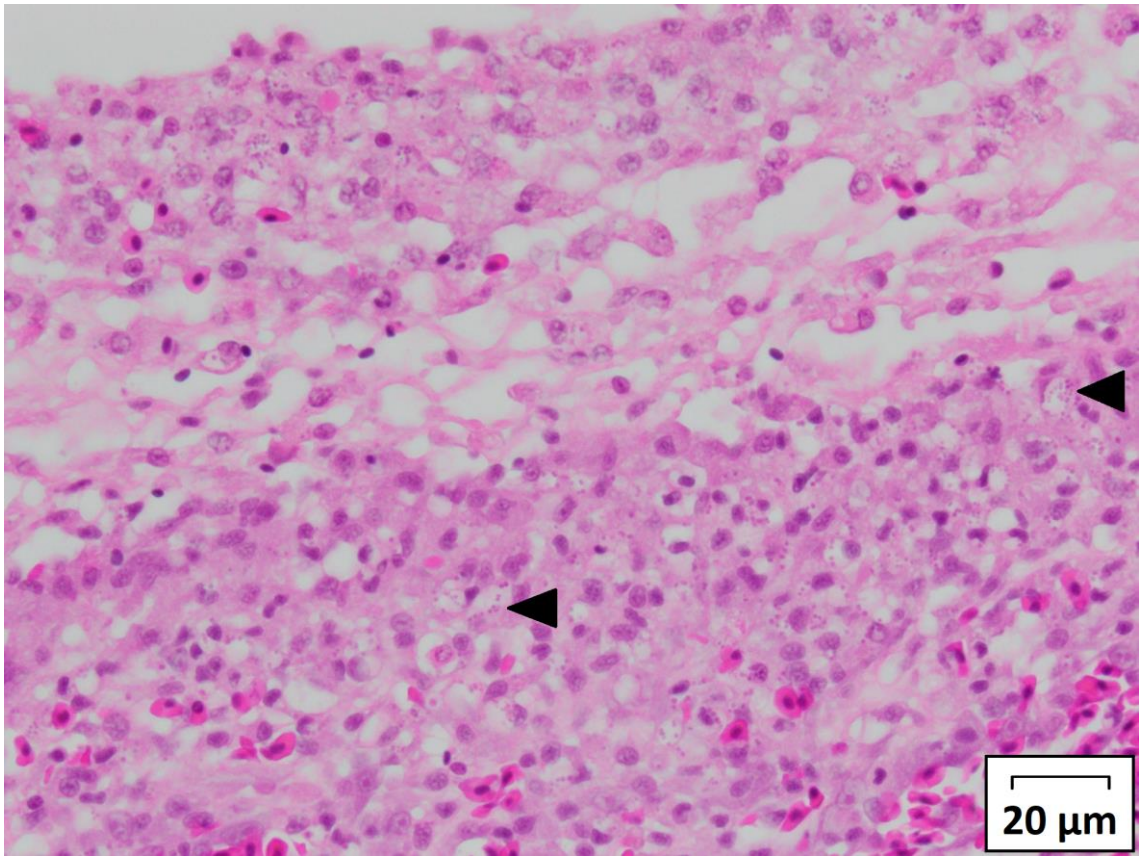


Figure 5.2 (H&E ×600). High power histomicrograph of Figure 5.1 showing numerous cocci forming chains (arrowheads) within the epicardium of *L. carponotatus.*

5.4 Discussion

The prevalence of *S. iniae* in wild aquatic species is largely unknown (Keirstead et al. 2014). The present study contributes to the knowledge of *S. iniae* in wild populations by identifying the first mass mortality event of wild reef fish in Australia associated with *S. iniae* infection, as well as the first recording of the bacterium in wild marine reptiles; flatback turtles and an olive sea snake.

Sudden death may be the first indication of a disease outbreak, with wild fish often succumbing to disease more rapidly than caged fish (Zlotkin et al. 1998, Bromage et al. 1999). Moribund fish from the Broome mortality event showed similar neurological symptoms to those reported in other *S. iniae* outbreaks, such as swimming erratically, circling and signs of disorientation (Perera et al. 1994, Ferguson et al. 2000). Fish also displayed exophthalmia similar to the disease outbreak in farmed barramundi at Lake

Argyle (Creeper and Buller 2006). However, commonly reported ascites and petechiae around the fins and anus associated with bacteraemia (Agnew and Barnes 2007, Buller 2014) were not observed. The histopathology was similar to other commonly reported pathological microscopic findings in *S. iniae* infection including visceral haemorrhage and systemic necrotizing inflammation such as fibrinous pericarditis/epicarditis and suppurative meningitis (Buller 2014, Keirstead et al. 2014).

Whilst the source of *S. iniae* associated with this outbreak is unknown, it is likely environmental factors contributed to this event. Prior to and during the Broome mass mortality event, a marine heat wave caused water temperatures to reach 38°C (normally 30.2°C) (Le Nohaïc et al. 2017), plus it was the hottest March on record for the north-west Kimberley coast (Bureau of Meteorology 2018). Although sea surface temperatures in the area during the January to April 2016 period were above average, there was a sustained sharp drop in ocean temperatures (approximately 2°C below the water temperature at the time) for nearly two weeks in late January to early February, possibly due to a deep-water upwelling event. As sudden marked decreases in water temperature are known to stress aquatic animals (Donaldson et al. 2008, Anderson et al. 2017), this may have caused altered immunity of animals in the region (Reid et al. 2022). Furthermore, as *S. iniae* prefers warmer temperatures (37°C) (Perera et al. 1994), it is possible anomalous temperatures could have precipitated the event, as noted in other outbreaks (Bromage et al. 1999, Ferguson et al. 2000, Rahmatullah et al. 2017). Algal biotoxins are also known to cause altered immunity in susceptible marine wildlife (Walsh et al. 2010), and a multitude of marine biotoxins were not excluded as contributory factors in this study. Potential sources of the *S. iniae* include substrate, carrier fish maintaining bacteria in the environment, or wild fish reservoirs (Zlotkin et al. 1998, Bromage et al. 1999, Nguyen et al. 2002). Previous outbreaks in wild populations have been associated with nearby fish farms (Zlotkin et al. 1998, Bromage and Owens 2002, Colorni et al. 2002). However, this scenario seems unlikely, considering there are no finfish aquaculture facilities in close proximity to the fish kill site and there have not been any known outbreaks in local aquaculture operations on the Kimberley coast.

In reptiles, *Streptococcus* spp. are part of the normal flora (Innis and Frasca 2017, Wellehan and Divers 2019). Despite a recent analysis of the microbiome of all sea turtle species including flatback turtles (Scheelings 2019), *S. iniae* has yet to be reported. *Streptococcus iniae* is typically a primary pathogen and not a contaminant (Trotter and Marshall 2003), therefore, the culture of *S. iniae*, in moderate to heavy growth in tissues including the kidneys, heart and liver of three turtles may indicate infection or at least a contributing factor, especially when consistent with the clinical picture (Morris et al. 2006). While it is likely the flatback turtle is affected by similar ailments as other sea turtle species, few reports of disease in flatbacks exist in the literature due to a scarcity of stranded specimen and, until recently, an inability to access and sample large populations of flatback turtles in foraging grounds.

As this outbreak occurred in a remote, unpatrolled area, collection of fresh specimens was difficult, with partial predation further deteriorating the carcass condition in many instances. While it is not uncommon to find stranded marine wildlife in the Broome area and frequently in an advanced state of decomposition due to the extreme environmental conditions, it is uncommon to find mass multi-species mortalities with significant species diversity (multiple piscine and reptilian taxa).

Moribund fish were collected and tested at the time of the Broome mortality event, however, turtle and sea snake samples were stored at Murdoch University at -20°C with no cryoprotectant for initially 12-18 months before being sent to DPIRD for culture. These suboptimal storage conditions were a serious limitation in the disease diagnostics for the flatbacks and sea snakes. However, recovery of some viable *S. iniae* cells after more than four years in storage was probably assisted by the thick peptidoglycan layer and lipoteichoic acid in the cell wall; any cryoprotectant ability offered by the host tissue; and the number of bacterial cells in the tissue prior to freezing (Major et al. 1955, Rice et al. 2015).

Despite the global nature of this disease in fish, it has not been detected in humans in the corresponding countries where fish kills have occurred, including Australia (Nawawi et al. 2008). There is significant confusion regarding this disease in the

literature (Baya 1996) due to missed identifications, misidentifications and reported variations in the biochemical profile hampering identification (Kusuda et al. 1991, Glibert et al. 2002, Bromage 2004). For example, *S. iniae* was incorrectly reported in the black flying fox (*Pteropus alecto*) (Yuniarti 2005) and further erroneously cited in additional publications (Agnew and Barnes 2007, Nawawi et al. 2008), but in fact the isolate was cultured from an ornamental aquarium flying fox fish (*Epalzeorhynchos kalopterus*) in WA (Dr N. Buller, DPIRD, personal communication, 20 August 2018). Due to the challenges associated with identification, it is possible that retrospective analysis of frozen samples, including "unidentified" streptococcal reports in humans or other species, could reveal additional cases (Baiano and Barnes 2009).

As a zoonotic disease, *S. iniae* also has public health risks. Infection with *S. iniae* in humans primarily causes cellulitis, but in more severe cases can cause endocarditis, meningitis, arthritis and toxic shock (Weinstein et al. 1997, Facklam et al. 2005). This bacteria usually enters the host through a breach in the skin barrier, as such good hygiene and use of PPE should be adhered to when handling infected animals, noting infected animals are not suitable for human consumption.

Emerging diseases are increasing in the marine environment, wildlife and in aquaculture (Harvell et al. 1999). As *S. iniae* is recognised for its sporadic outbreaks and as an important emerging aquatic animal pathogen, this highlights the importance of ongoing monitoring for disease and the timely reporting of morbidity and mortality events. Although *S. iniae* was detected by culture and/or PCR in four flatback turtles and one sea snake, future research of fresh samples is required where culture and histopathology can be conducted to determine the significance of the finding of *S. iniae* in marine reptiles.

CHAPTER 6

General discussion

6.1 Summary of key findings

This study assessed the current health and disease status of sea turtles in WA over a three-year study period. The aims of the research were to:

(i) Establish a health baseline for sea turtles in WA; and

(ii) Establish causes of morbidity and mortality of sea turtles in WA.

These aims were achieved through a combination of health assessments and disease investigations, and by addressing the study's key objectives; which were to:

(i) Perform health assessments on populations of nesting and foraging flatback turtles; and

(ii) Undertake disease investigations in five stranded sea turtle species.

The key findings from these health assessments and disease investigations are reported in the three data chapters (Chapter three, four and five).

In Chapter three, blood RIs were developed for flatback turtles, previously the only sea turtle species without RIs (Stacy and Innis 2017). These blood RIs provide an indication of systemic health and are central to any baseline health survey (Deem et al. 2001, Herbst and Jacobson 2003). Using health assessments, the first blood RIs were developed for nesting flatback turtles, while RVs were established for foraging flatback turtles for the first time. These RIs/RVs were developed following the ASVCP guidelines, implementing outlier detection methods, exclusion criteria and appropriate statistical analyses (Friedrichs et al. 2011). Separate RIs/RVs were developed for life stage (i.e., nesting or foraging) due to significant differences for blood analytes between life stages. Other significant differences were also detected in analytes for boundary conditions such as rookery, season and sex. While RIs (and RVs) have been established for other species in Australia, including green, loggerhead and hawksbill turtles (Flint et al. 2010b, Flint et al. 2010c, Whiting et al. 2014b), few flatback turtle haematologic and plasma biochemical values exist for comparison with our study's flatback RIs/RVs (Sperling et al. 2007, Guinea 2016, Scheelings et al. 2020). Results were overall similar to flatback and other sea turtle RI and RV studies, however,

due to different pre-analytical, analytical and post-analytical factors in the various studies, comparisons should be interpreted with caution (Stacy and Innis 2017). For example, extrapolation of RIs is difficult due to the different methodologies used in the different studies, where methodologies include pre-analytical factors such as anticoagulant type (Harr et al. 2005) and plasma storage conditions (Hawkins et al. 2006); analytical factors such as analyser type (McCain et al. 2010) and the specific laboratory used to develop the RIs (Friedrichs et al. 2012); and post-analytical factors such as outlier detection techniques and statistical analyses for the establishment of RIs. In our study we found most blood analytes tested in the laboratory were significantly different from results for the same analytes tested in the field using the i-STAT blood analyser. Further, aside from the aforementioned factors, biological variation including both intra-individual and inter-individual variability can affect blood values, especially relevant to ectothermic sea turtles strongly influenced by both intrinsic factors (e.g., species, sex, age-class, physiological status) and extrinsic factors (e.g., season, habitat, epibiota load, and environmental conditions such as temperature). Through this baseline health research, a novel *Haemocystidium* intraerythrocytic parasite was also discovered in flatback turtles (and in green turtles, data not shown), specifically in the foraging life stage. Although intraerythrocytic parasites have been reported in loggerhead turtles from the Indian Ocean, these parasites are yet to be globally recognised as a pathogen of sea turtles (Trocini 2013).

In Chapter four, the first disease investigation for causes of morbidity and mortality of stranded sea turtles in WA was developed through the use of detailed veterinary investigations such as clinical examination, necropsy, histopathology and parasitology for five sea turtle species (including green, loggerhead, flatback, hawksbill and olive ridley turtles). Globally, causes of sea turtle strandings are frequently unknown, due to a lack of detailed veterinary investigations (Flint et al. 2010d, Stacy et al. 2017b); an issue which also exists in WA and other parts of Australia (Gordon 2005, Raidal et al. 2006). Previous morbidity and mortality studies of stranded turtles in this state have involved a total of five individuals, including green and loggerhead turtles (Raidal et al. 1998, Raidal et al. 2006). While similar studies in other parts of Australia have had

larger sample sizes (i.e., >100), these studies have been limited to green turtles (Gordon 2005, Flint et al. 2010d). In the present study, natural disease-related causes of mortality occurred more frequently than direct anthropogenic causes, with parasitoses the most frequently occurring natural disease, which was similar to the findings of other studies in WA and Australia (Raidal et al. 1998, Flint et al. 2010d). As expected, spirorchiidiasis was the most common cause of mortality in stranded turtles in our study with a disease prevalence of 93.2%; comparable with previous reports of prevalence of 98% in Queensland (Gordon et al. 1998a). Our research also reported spirorchiidiasis in the flatback turtle for the first time, and neurospirorchiidiasis in the olive ridley turtle – not previously reported in this species (Santoro and Morales 2007, Jerdy et al. 2016, Chapman et al. 2019). Aside from the *Neospirorchis* sp. eggs found in the flatback turtle brain (and the adult *Hapalotrema* spp. found in the flatback turtle WT82 liver wash outside the study period), all other species and genera of spirorchiid in green, loggerhead and hawksbill turtles in our study had previously been detected in the corresponding species (Chapman et al. 2019). As expected, spirorchiidiasis occurred more frequently in the north region when compared to the south region, likely related to the presence of intermediate hosts in these warmer northern areas (Flint et al. 2009b). Despite the apparent low detection rate of spirorchiids through organ washes, this technique was more successful than histopathology or necropsy for the detection for adult spirorchiids. Unusual and emerging diseases were also reported, including microsporidial myopathy, salt gland adenitis, gout and pseudogout, as well as the detection of a novel zoonotic bacterium in reptiles (see next paragraph) (Orós et al. 2005, Oros et al. 2011, Martinson et al. 2018). Considering that diseases in the marine environment are predicted to rise with increasing anthropogenic pressures (Ward and Lafferty 2004, Hoegh-Guldberg and Bruno 2010), detection of new and emerging diseases is of significance to the global knowledge of sea turtle diseases; and for understanding and mitigating disease threats to sea turtle populations (Stacy et al. 2017b).

In Chapter five, we report the first record of a multi-species mass mortality event involving sea turtles, sea snakes and fishes in WA (Young et al. 2020). While

environmental factors, including a marine heat wave (Le Nohaïc et al. 2017) was likely to have contributed to the event, our disease investigations detected the zoonotic bacterium, *S. iniae*, in flatback turtles, sea snakes and multiple fish species. While some sea turtles, sea snakes and fish had positive culture and positive PCRs, bacteria-associated pathology was identified only in the fish samples (from freshly euthanised fishes). Unfortunately, autolysis and freezer issues precluded positive bacteriological results in some sea turtle, sea snake and fish specimens, and identification of pathology in sea turtle and sea snake samples (Morris et al. 2006, McAloose et al. 2018). While certain variables – such as high environmental temperatures – cannot be controlled, these sample issues highlight the importance of a coordinated stranded turtle reporting network in a large state such as WA, and the importance of the timely recovery of cadavers, as well as collaboration with veterinary diagnostic specialists to ensure appropriate storage of cadavers and samples in future mass mortality events. As this is the first report of *S. iniae* in reptiles, this further strengthens the importance of disease investigations of stranded sea turtles; particularly considering our limited understanding and knowledge of disease threats facing sea turtles (Flint et al. 2010d).

6.2 Limitations

Western Australia is a large state, with an often rugged and remote 21,000 kilometres of coastline. As such, there were significant logistical and financial challenges in undertaking this health and disease investigation study, as is the case in other wildlife health studies (Ryser-Degiorgis 2013). While some sample collection and diagnostic testing occurred in the field or at regional locations, most diagnostic procedures were performed in Perth. Stranded turtles, cadavers and biological samples collected in the field were transported long distances (up to 2200 km) for diagnostic testing, including laboratory haematology and biochemistry, necropsy, histopathology, and parasitology.

For the health baseline study, large distances and extended transportation times to the laboratory in Perth made time-sensitive analyses particularly challenging. While

plasma samples can be stored frozen, and blood films can be stored at room temperature, whole blood samples for haematological analysis (i.e., PCV, RBCC, Hb and other calculated values) must be refrigerated and arrive at the laboratory within 48 hours from collection for analysis (Fudge 2000a). Due to difficulties in transporting blood samples from remote study sites, the cut-off time point for inclusion in the analysis was increased from 48 hours to 60 hours to reflect these real-world scenarios. Although significant differences for the various blood analytes were rarely detected for the different time groups (i.e., <48 hours and 48-60 hours), further investigation would be worthwhile to confirm the results of these analyses; such as by testing a larger sample size or running serial testing on the same sample for the different time groups (Eisenhawer et al. 2008). The use of different analysers for the haematological component for the different life stages, i.e., Cell-Dyn 3700 for nesting and Sysmex XN-1000 for foraging groups, was another limitation of the health study, and comparison of the haematological results for the different life stages should be made cautiously.

Limitations of the disease investigations were associated with accessing fresh cadavers, as well as storage, transportation and environmental conditions. Most turtles stranding in regional areas of WA were usually found dead, with cadavers in deteriorated condition (WAStD accessed July 2021), especially in the northern parts of WA. These northern areas experience extreme environmental conditions; for example, a marine heatwave with water temperatures of 38°C occurred near Broome during the mass mortality event in March 2016 (Le Nohaïc et al. 2017) (Chapter five). Cadavers found in regional areas were usually also frozen prior to transport, due to the logistics of transportation in remote areas. As such, freezing and autolysis were both significant issues in this study, having major impacts on diagnostic capabilities (Stacy et al. 2017d, Cook et al. 2020). Furthermore, adult sea turtles are large animals (approximately 100 kg) and storage space in freezers can also be a limiting factor.

Another limitation in our study – which is applicable to any field-based research – is the highly variable nature of field work; which is strongly affected by environmental conditions, and is very different from working under laboratory conditions (Friedrichs

et al. 2012). In the northern parts of the state, extreme weather such as cyclones, storms, extreme heat and wind can impact on the research and affect the results. For example, transportation delays may cause various issues, including temperature-related sample artefacts such as haemolysis (Eshar et al. 2018); which artefactually increase most biochemical analytes (Stacy et al. 2019b) and decrease haematological values (Fudge 2000a). However, where necessary, weather- and environment-affected samples were removed from the analysis.

Cost can be another major limiting factor of wildlife research (Karesh 1993, Ryser-Degiorgis 2013). As the veterinary diagnostics involved in health and disease investigations (such as haematology and biochemistry analyses, or histopathological analysis) require the use of expensive diagnostic machinery and the expertise of veterinary diagnostic specialists, testing is expensive. Remote work and transportation costs associated with remote locations is also expensive (Ryser-Degiorgis 2013). Field work which requires vessels, such as foraging flatback turtle health research, is weather dependent and particularly expensive, requiring extensive logistics and planning.

As with most wildlife studies, there are limitations with access to wild animals, and limitations associated with study methodologies (Ryser-Degiorgis 2013). Sample size was a limiting factor for some aspects of our study; for example, in the foraging flatback baseline health research. If ASVCP guidelines are followed, the minimum recommended sample size for RIs is 120 (Friedrichs et al. 2011). While our research sampled 148 nesting flatback turtles, we only sampled 50 foraging flatback turtles. To address this, RVs were presented for foraging flatbacks rather than RIs. Like other wildlife studies, some parts of our study used opportunistic and convenience sampling, potentially causing selection bias (Friedrichs et al. 2012, Ryser-Degiorgis 2013). For example, most stranded turtles were from human-populated or patrolled areas, which biased the distribution of stranding sea turtles. In contrast, selection bias was removed by semi-randomly sampling the next turtle encountered for nesting (and foraging) turtles health assessments. However, it is possible some foraging turtles were more

easily captured due to ill-health. There may also be limitations with the types of investigations undertaken. For example, although detailed veterinary disease investigations are significantly more likely than stranding surveys to be able to provide a diagnosis (Stacy et al. 2017b), disease investigations have their own limitations; including cost and low sample sizes, if diagnoses are unable to be reached, or if sublethal impacts on fitness, reproduction and survival are overlooked (Gordon 2005).

Another limitation of this study and any future stranded turtle studies in WA is the incomplete reporting of stranded sea turtles in the state. Although reporting in WA is improving, many stranded turtles encountered in WA are unreported. In addition, many stranded turtles would remain undiscovered, due to the largely remote and unpatrolled coastline meaning actual numbers of stranded turtles in WA are unknown. Similar to other stranded turtle studies, in our study there was a bias towards stranded turtle reporting in populated and patrolled areas (Chaloupka et al. 2008); leading to a lack of study animals from the remote and sparsely populated regions of the East Kimberley, Mid-West, and the Goldfields District east of Esperance (Figure 4.3). Furthermore, the number of stranded turtles reported will only be a small proportion of the total number of turtles that die at sea (Stacy et al. 2017b). In US studies, it has been estimated that reported numbers of stranded turtles represent only 10-20% of total mortalities (Epperly et al. 1996, Hart et al. 2006, Stacy et al. 2017b). However, the relevance of the estimates of sea turtle deaths at sea in these studies (Epperly et al. 1996, Hart et al. 2006) to at-sea mortalities of east Indian Ocean sea turtle populations is unknown, as these studies have been conducted in different locations under different conditions (such as oceanographic currents) (Stacy et al. 2017b). Nonetheless, as at-sea mortalities in WA exist, actual stranding and mortality numbers are likely to be significantly higher than current estimates.

Wildlife health investigations are challenging as there is a paucity of literature regarding health and diseases of wildlife as compared to domestic species (Deem et al. 2001, Ryser-Degiorgis 2013). As wild animals will lack an individual history, establishing an individual's health status or determining cause of morbidity and

mortality is difficult (Friedrichs et al. 2012). Despite the continued increase in knowledge of reptilian health and disease, there is also much less known about the health and disease of reptiles compared to mammals (Zimmerman et al. 2010, Ariel 2011, Barraza et al. 2021). For example, many diagnostic tests have not been validated in reptiles (Campbell 2014), and blood analytes tested may not be applicable to reptiles (Herbst and Jacobson 2003). Similar to other wildlife species, there also are knowledge gaps in natural history, basic physiology and anatomy, as well as disease and health in sea turtles that currently inhibit our ability to confidently perform health assessments in sea turtles that accurately reflect the health status of an individual or population (Herbst and Jacobson 2003, Rhyan and Spraker 2010, Ryser-Degiorgis 2013). Wildlife will also mask disease as a survival strategy, which is especially true for reptiles given their stoic nature and the subtlety of clinical signs (Flint et al. 2010d, Rhyan and Spraker 2010).

The complexity of the sea turtle life cycle could also be considered a limitation if not taken into consideration in assessing the results. For example, the stranding location does not indicate the turtle is a resident of the area (i.e., foraging grounds), or that the cause of the stranding occurred in the area, as the turtle may have been undertaking a breeding migration, or it may have arrived to the area due to oceanic currents. Similarly, haematological and biochemical blood results from turtles sampled at the nesting site will often reflect the environment in which the turtle spends the majority of its time (e.g., foraging grounds). Furthermore, resident turtles at foraging grounds are of mixed genetic stock, with turtles from one resident area utilising beaches within a 2000 km radius. Therefore, at the time of blood sampling, foraging turtles may have been preparing for breeding or returning from migration and may show major differences in their blood values depending on the stage of their life cycle and the distance to the nesting beach.

One final limitation of this research is the issue of shifting baselines, whereby a baseline gradually, sometimes undetectably, shifts over time (Deem and Harris 2017). This results in inappropriate reference points for assessing population impacts (Pauly

1995). Various types of study sites were utilised for our baseline health study (i.e., undeveloped, industrial and town sites). While the undeveloped site would be expected to have the least human impacts, it is unlikely that any location on the planet is unaffected by humans. Consequently, the baseline is the current baseline for these localities, emphasising the urgent need for the development of baselines and serial health monitoring (Deem and Harris 2017). This limitation can be accounted for by collecting a subsample of healthy animals to compare changes in blood and health values over time or for different regions, and will provide an idea of any shifts in normal values. As human-induced impacts continue to place increasing pressure on wildlife in their environments, additional harmful changes to wildlife health are expected and imminent.

6.3 Recommendations

6.3.1 State management of stranded sea turtles

As the agency responsible for the management of sea turtle stocks in WA, DBCA is responsible for developing state guidelines, and other legislative documents such as stranded turtle reporting protocols. State-specific guidelines need to be developed to support current state legislation, similar to those in other states such as *the Code of Practice for Injured and Sick Sea Turtles and Sea Snakes in NSW* (NSW Department of Planning 2020). Guidelines should be developed in consultation with a range of stakeholders including wildlife veterinarians to include specific information for rescue, transportation, triage, housing, quarantine, rehabilitation, treatment, reporting, welfare and euthanasia of stranded sea turtles. Once developed, these guidelines should be disseminated to metropolitan and regional DBCA offices, and to other key personnel including Perth Zoo veterinary department, rehabilitation centres and wildlife carers, veterinary clinics (via the Veterinary Surgeons Board), researchers, councils, ranger groups and industry. Appropriate instruction and training should accompany the guidelines to ensure standardisation across the state. It is important that any legislation related to stranded turtles is enforced by the agency

developing the guidelines, to ensure the best outcomes for individual welfare and population conservation.

Sea turtles have specialised husbandry requirements (Stamper et al. 2017), and practical training is required for all DBCA staff, wildlife carers and other key personnel caring for stranded sea turtles, however, there is currently no agency responsible for this training. Nonetheless, key personnel involved with stranded sea turtles should be trained in rescue, transport and triage of sea turtles. Examples of key messages which need to be clearly communicated to relevant stakeholders include turtles should not be transported in water, cold turtles should not be fed or medicated, turtles should be transported in secure and appropriate containers, and containers should be padded (especially important in emaciated turtles prone to avulsions and other injuries) (Innis et al. 2017a, Manire et al. 2017c, Norton et al. 2017). While lifting robust turtles by their flippers generally does not cause problems, this technique should never be used in debilitated, stranded turtles due to the unacceptable risk of fractures and other musculoskeletal trauma. Debilitated turtles should also never be placed upside down (plastron up) as there is a risk of the plastron bones penetrating internal organs due to a lack of the supporting connective tissue (Manire et al. 2017c). Training on how to perform a simple procedure, such as placing a turtle in water, also needs to be provided. For example, a turtle should be placed only in a shallow bath to avoid drowning (especially moribund animals) and warmed up slowly in water close to the turtle's core body temperature (see Section 6.3.2 below). A specialised thermometer is required to accurately measure a turtle's body temperature, as reptile temperatures often read outside the range of standard thermometers (lowest standard thermometer reading of 32°C is hyperthermia in a sea turtle) (Norton et al. 2017). However, generally, a sea turtle will only be within a few degrees of the ambient temperature of the medium (water or air) where it is located (Sato et al. 1995). To perform basic triage, required equipment includes crates or plastic clam shell pools, a heat source (such as a heat lamp or a thermostat-controlled heating mat), as well as temperature monitoring equipment for the water and turtle (such as an infra-red

temperature measuring device e.g., Raytek®gun, Raytek Corporation, North Carolina, US) (Norton et al. 2017).

Although WA is a large state with vast areas of remote coastline, the current completion rates for stranded turtle reporting forms still need improvement. In our study, many turtles did not have a stranding form completed, or forms were incomplete with important data missing. DBCA is responsible for training DBCA staff and other key personnel to improve completion rates of stranded turtle reporting forms and convey the importance of reporting strandings. By providing training on the use of the stranding form, including species identification and external examination such as body condition scoring, decomposition scoring and recording of epibiota, the data collected will be significantly more useful. The stranding form also needs to be updated to improve the quality of data collected, for example including check boxes for the presence or absence of important variables such as positive buoyancy. There are currently multiple different stranded turtle reporting forms in circulation in WA, and once the form is updated, a single form needs to be used to ensure consistency of reporting. It is also recommended nesting beach or in-water turtle monitoring activities formwork be updated to include standardised physical examination records such as body condition score and documentation of external abnormalities.

Additional educational training workshops in necropsy and pathology need to be provided to key personnel by wildlife veterinarians or other trained wildlife health professionals, to improve the general understanding of these areas and increase disease awareness (Ryser-Degiorgis 2013). Examples of training include temperature effects on decomposition and effects of refrigeration and freezing on diagnostic samples. In our study, reaching a diagnosis of *S. iniae* in sea turtles found during a mass mortality event was impeded by decomposition of cadavers (Chapter five). While this event occurred during extreme temperatures in the wet season, multiple freeze thaw cycles may have played a role in worsening autolysis caused by a freezer malfunction and removal of cadavers from the freezer at a separate time from necropsy, for measurements and sample collection. To improve sample and cadaver storage

conditions, ideally, key personnel around the state would be set up with equipment solely for the recovery of dead turtles (i.e., equipment separate to that used for live turtles to reduce the risk of disease transmission), including eskies and frozen ice bricks/water bottles to slow the autolysis process and improve sample quality. Specimen and samples transported to Perth from regional areas should be transferred with adequate cooling, to ensure samples stay frozen/chilled until arrival. Staff at DBCA and other key personnel should also be trained in sea turtle necropsy. If a dead turtle is found and a necropsy will not be performed, then trained personnel need to conduct a detailed external examination and report findings on the stranding form as explained above.

Although direct anthropogenic impacts caused stranding less frequently than natural disease in WA, direct human impacts can often be mitigated (Witherington 2017). One example of a direct anthropogenic impact is vessel strike; and although generally fatal, it is not thought to cause major population declines of sea turtles in Australia (Commonwealth of Australia 2017b). However, turtles which die acutely are less likely to be found as these cases generally sink initially, floating only once the carcass putrefies, potentially arriving on the shore in advanced decomposition (Epperly et al. 1996). Therefore, even though only two fatal vessel strikes were found in this study (2.7%), the impact of vessel strike could be far greater as these animals are less likely to be retrieved due to being in an unsuitable condition for necropsy, and one of these two turtles was a pre-ovulatory breeding turtle and of high reproductive value to the population. Further, considering the significance of the removal of a single adult from the population, mitigation strategies should consider water use zoning where turtle habitat and high levels of vessel traffic overlap. For example, ‘go slow’ zones to reduce watercraft speeds have been found to significantly decrease mortalities (Work et al. 2010). Other management strategies to mitigate human impacts include seasonal closures of nesting beaches and adjacent waters, to protect critically important breeding animals from injury and/or disturbance (Commonwealth of Australia 2017a). Additionally, increased education of recreational and commercial operators to reduce vessel strike, and reporting vessel strikes and sightings of injured turtles would

improve the understanding of hot spots and seasonality of incidents, assisting managers with zoning (Commonwealth of Australia 2017a). Another example of a future threat for WA requiring a mitigation strategy is oil spill response, for which a state response plan is currently in development.

While customary take was responsible for the mortality of several turtles in this study, including one sub-adult, traditional hunting is a culturally significance practice and is managed under traditional customs and lore. However, considering the conservation importance of a single adult turtle, especially for threatened sea turtle populations, it would be preferable if adults, especially breeding turtles, were avoided in customary take. Sustainable management practices may be implemented by engaging with local communities, development of planning documents such as the Yawuru Nagulagun/Roebuck Bay Marine Park Joint Management Plan (Department of Parks and Wildlife 2016) or through indigenous consultative workshops such as the recent Saltwater Advisory Group Forum held in Broome (1 December 2021) by nine Kimberley Saltwater Native Title Groups through which the 10 year Kimberley Indigenous Turtle and Dugong Initiative was established.

While management of natural threats is generally not feasible, some mitigation is possible. For example, beaches in south-west WA could be monitored for stranded turtles in winter, especially after storms. Such informal programs exist in the Bunbury region, coordinated by Dolphin Discovery Centre (DDC) (J. Tierney, DDC, personal communication, 11 June 2019). Many of the stranded turtles found in the South-West during winter are cold-stunned post-hatchlings which are usually loggerhead turtles, although flatback and hawksbill turtles may occasionally be found. These cold-stunned turtles are frequently released after a period of rehabilitation; highlighting the value of stranded turtle beach surveys. If additional beach surveys were to be undertaken, location and effort (i.e., time spent) should also be logged, to monitor changes associated with cold-stunned turtle stranding over time.

Although much data on sea turtle strandings in WA have been collected over the years, few reports or publications have been produced. There is currently a new database in

production (WAStD) to register stranded sea turtles (and other marine life), for which a great deal of time has been invested over the past five years. The database is continually evolving and has enormous potential, and it can be utilised to perform analyses and produce reports. It is recommended that annual sea turtle stranding reports are produced in WA, like in Queensland (Haines and Limpus 2000, Greenland et al. 2004).

6.3.2 Veterinary management

The following recommendations are for veterinarians responsible for the care and treatment of sea turtles in WA. As the primary veterinary hospital treating sea turtles in WA, Perth Zoo Veterinary Hospital already implements most of the following recommendations.

Clinical examination and triage

- An initial assessment including triage should be performed on arrival. This should include weight, body condition score, cloacal temperature, fluorescein stain for ocular ulcers, and examination for any other major abnormalities, which should be addressed as required (Tristan and Norton 2017).
- Once the temperature of the sea turtle has been checked, the turtle should be placed in a shallow freshwater bath at a temperature no more than 3°C above cloacal temperature, with any water temperature changes made gradually (maximum change of 3°C per day, unless severely hyperthermic) (Norton et al. 2017). Salinity changes should also be done gradually (maximum 33.3% change per day). Freshwater baths have been shown to help with rehydration (Norton 2005) and with removal of epibiota. Hunt et al. (2019) have also shown that placing a turtle in salt water significantly improves recovery rate after long transportation. Considering the stress associated with transport, and the long distances turtles may travel within the state, a shallow warm bath is also recommended prior to travel. While freshwater baths are critical at the early stages of treatment, leaving turtles in freshwater for as short as four days can cause mortality (Field et al. 2017, Innis et al. 2017a).
- A standardised sea turtle diagnostic examination should be performed once stable and should include a detailed examination, neurological examination (Chrisman et al. 1997, Harms et al. 2017, Tristan and Norton 2017), buoyancy assessment including degree of buoyancy and tilt direction, respiration effort and rate, epibiota assessment including type (for example burrowing barnacles, macroalgae, leech eggs), grade, coverage and distribution. This standardised diagnostic examination should preferably be performed by day three.

- Monitoring of sea turtles in care should include daily records of mentation, appetite, defecation, buoyancy and activity. A treatment record should be kept with accurate records of treatment type, dose and frequency.

Diagnostic testing

- Radiography should be performed on each turtle admitted to a veterinary clinic.
- Blood should be collected for in-house PCV, TPS, glucose and a blood film for TWCC and differential WCCs (Norton et al. 2017). If funding is available, blood should be collected into Li-Hep tubes for haematology and biochemistry (CK, AST, BUN, uric acid, glucose, sodium, potassium, chloride, total protein, albumin, globulin, calcium, phosphorus) and performed in the laboratory. Blood should be kept refrigerated post-collection until sending to the laboratory. If blood is unable to be analysed the same day, whole blood should be aliquoted first, then blood should be centrifuged and the plasma separated. Subsequently, a blood film, whole blood, and separated plasma should be sent for analysis. A drop of Li-Hep blood on filter paper can also be stored at room temperature for potential future molecular studies.
- An in-house faecal examination should also always be performed in stranded sea turtles in WA, considering the high rate of parasitoses as a cause of morbidity and mortality. If faecal floatations are not performed in-house, then faecal samples should be sent to the laboratory for parasitology. The challenge with faecal examination in sea turtles is the difficulty with distinguishing between trematode eggs which are pathogenic (spirorchiids) and those which are non-pathogenic (e.g., gastrointestinal trematodes) (Prof. D. Blair, James Cook University, personal communication, April 2017). However, parasitology training could be provided to staff to assist with identification of trematode eggs, to facilitate in-house testing and reduce costs associated with out-sourcing of parasitological testing.

Veterinary treatment

- Considering the high rate of parasitoses in stranded sea turtles, anthelmintics play an important role in treatment regimens. Praziquantel can be used for both trematodes and cestodes. For cestodiasis, the standard dose rate of 8 mg/kg two weeks apart is used. However, for spirorchiidiasis, an increased dose rate of 25 mg/kg orally for three days is recommended (Dr D. Mader, Marathon Vet Hospital, Florida, 11 October 2017). As spirorchiidiasis is not found in oceanic turtles, treatment for spirorchiidiasis is not required in this life stage. However, as GI cestodiasis was particularly common in cold-stunned post-hatchling loggerheads, treatment for cestodiasis at the standard dose rate is recommended for this oceanic life stage.
- While some turtles were found with a moderate to heavy burden of nematodes, nematodiasis did not occur as commonly as cestodiasis or trematodiasis. It is therefore recommended that treatment with a nematicide be commenced only

after a positive faecal floatation (additional training is not required for nematode egg identification as nematode eggs are readily identifiable).

- Although mixed GI endoparasitoses were common, not all contributed to morbidity and mortality, and therefore anthelmintics should only be used when necessary.
- Although spontaneous regression of FP tumours may occur, surgical removal (or staging and debulking) of FP tumours can be performed with good success using scalpel excision, radiosurgery, cryosurgery, CO_2 laser, or a combination (Di Bello et al. 2017). However, treatment of FP turtles requires particular attention to quarantine and FP turtles should be housed separately in tanks with separate filtration systems to prevent the spread of infectious agents such as ChHV5 associated with this disease (Stamper et al. 2017).

Additional diagnostic testing

- Electrophoresis could be used for protein fractions, as albumin measurement using the bromocresol green method is not validated in reptiles (Heatley and Russell 2019a).
- CT is a potentially useful diagnostic imaging modality for cases unresponsive to treatment, with unknown aetiology or to assess prognosis (Pease et al. 2017).
- Endoscopy could be used to diagnose severe spirorchiidiasis cases, identify sex, or to assess other internal pathology (Divers et al. 2017).
- Blood gases are particularly useful as prognostic indicators (Innis et al. 2007, Keller et al. 2012, Stacy et al. 2013).
- Microbiological culture swabs and blood culture could be used to identify septicaemia and associated micro-organisms plus antimicrobial susceptibility or resistance (Innis et al. 2017a).

6.3.3 Rehabilitation management

To rehabilitate sea turtles, all rehabilitation centres and wildlife carers are required to be licenced by DBCA. A triage approach should be taken including assessing for likelihood of a healthy recovery. All turtles in rehabilitation should receive a veterinary assessment, radiography, in-house bloods and a faecal floatation. Detailed monitoring of turtles in care should include daily recording of mentation, appetite, defecation, activity and buoyancy; and these records should be made available for DBCA when requested. Unfortunately, some turtles treated at rehabilitation centres in this study did not have treatment regimens recorded, which meant the efficacy of treatment could not be assessed; and these animals had to be excluded from the various

analyses. It is therefore imperative to record all treatment of turtles during rehabilitation on treatment records. Generally, rehabilitation animals should not be on public display (*Biodiversity Conservation Regulations 2018*). If the facility has captive animals, wild turtles should be housed in a completely separate system to avoid the transmission of infectious diseases between animals, and, most importantly to reduce the risk of released animals introducing disease into wild populations.

6.3.4 Future research

Future research projects on sea turtle health and disease in WA should focus on the most significant knowledge gaps. Development of baselines is one such important research area for WA sea turtles. As ecotoxicology is recognised as an increasingly important field in sea turtle health, toxin and heavy metal baselines should also be established, as well as investigations into the effects of various toxins. These toxicological investigations can be done without the need for animal experiments, through the novel use of sea turtle cell culture (Finlayson et al. 2016). Other baselines for protein fractions using electrophoresis instead of traditional methods should also be developed (Flint et al. 2015c). Considering the high levels of parasitoses amongst WA sea turtles, further parasitology studies should be developed to perform detailed parasitological investigations, especially for pathogenic parasites such as spirorchiids. Given that blood RIs can vary between different locations and different populations (Whiting et al. 2007a), it would be worthwhile to develop blood RIs for other sea turtle species in WA, such as green turtles and olive ridley turtles (using results from blood samples collected during this PhD research), as well as blood RIs for foraging flatback turtles.

Another important area of future research is sea turtle disease. Considering that the majority of turtles in this WA study were affected by natural disease causes, rather than anthropogenic causes, further investigations into aetiology and pathogenesis of sea turtle disease is warranted. For example, investigations should be undertaken into disturbances of the host-pathogen-environment relationship, and the potential role of indirect anthropogenic threats on individual and population health; such as low-level

long-term toxin exposure and the varied and serious potential climate change-related impacts. Epidemiological studies to identify levels of disease in populations can be started by implementing disease surveillance programs for all sea turtle species, especially flatbacks, with investigations targeting important diseases such as fibropapillomatosis and coccidiosis. By monitoring disease levels in populations, changes can be identified over time, and sea turtle managers can be informed of any major changes (Deem and Harris 2017). Furthermore, considering the challenges with identifying health and diagnosing disease using sea turtle blood values, improved diagnostic techniques should also be developed (March et al. 2018). One such example is the development of novel biomarkers, such as inflammatory markers, which could be another area of research focus (Marancik et al. 2021, Melvin et al. 2021), with assays for the acute phase protein marker, haptoglobin, currently underway (Dr G. Rossi, Murdoch University, personal communication, July 2021). Development of serological bioassays to detect spirorchiid infection, such as indirect ELISA (Work et al. 2005), would be useful for ante-mortem diagnosis of spirorchiidiasis and to better understand association between clinical signs, pathology and parasite burden (Chapman et al. 2019). It is also recommended that specific-ChHV5 primers are used in future fibropapillomatosis studies in WA (Jones et al. 2020, Mashkour et al. 2021) due the unsuccessful attempts of herpesvirus detection with the generic herpesvirus primers used in this study.

Investigations into the causes of sea turtle stranding in the state through necropsy should remain an important area of future research. According to Gordon (2005), a thorough necropsy can be as useful as histopathology. Necropsies and histopathology should be prioritised in cadavers in good condition (i.e., D1 and D2). Necropsies could still be performed in D3 and D4 animals; however, histopathology would only be worthwhile in groups with low representation, such as healthy animals, acute causes of mortality, endangered species like hawksbill turtles, and species in which less is known, such as flatback turtles, as well as in mass mortality events, and turtles from understudied regions and industrialised areas. Improving the stranded turtle reporting

network would improve access to fresh cadavers and opportunities for necropsy (and histopathological examination).

Identifying the final cause of mortality in sea turtles can be challenging, especially in autolysed cases, as similar to other wildlife, stranded sea turtles typically present with multifactorial disease (Van Bressem et al. 2009, Osinga et al. 2012, Flint 2013, Griffith et al. 2013, Ryser-Degiorgis 2013, Manire et al. 2017c). Fresh turtle cadaver cases are particularly useful as the cause of death can usually be attributed, given that cases are not suffering from multiple chronic diseases (Stacy et al. 2010a). Necropsies of clinical cases which die should remain a priority, as these cases are frequently in good condition and have a clinical history; and thus ante-mortem findings and post-mortem findings can be correlated. For example, praziquantel treatment is an important therapy in post-hatchlings, considering the extensive pathology associated with cestodiasis; however, as no post-hatchlings completed a course of praziquantel in this study, drug efficacy could not be assessed or correlated with pathology. It is recommended that future post-hatchling mortality cases should have thorough gastrointestinal histopathology undertaken (noting GI tract autolyses rapidly and this should be prioritised in fresh cadavers), and gastrointestinal cestode burdens should be compared with praziquantel treatment, to ensure the regimen's efficacy.

Considering how little is known about flatback turtle health and disease compared to other sea turtles (Deem and Harris 2017), a research focus should continue in this species. Ongoing health assessments of flatback turtles, including blood collection, allow for the continued monitoring of population health over time. Increased efforts to perform health assessments in certain groups of flatback turtles with low representation, such as males (especially breeding males), and juveniles, should remain a priority. While RIs in nesting flatback turtles have been developed, RIs for foraging flatback turtles should also be established. Given the small number of necropsies performed in flatback turtles, necropsies in this species should also be prioritised.

Clinical cases can serve as a very important source for research. If a clinical case is a designated research case, consistency is important when collecting ante-mortem data. For example, ideally research clinical cases should have blood collected as soon as possible following arrival, and prior to treatment. Therefore, if an animal dies shortly after arrival, blood has been collected. However, blood should only be collected if the animal is stable. Provided that ethics permits have been granted, various blood samples can also be collected, depending on the type of testing; including Li-Hep plasma, whole blood and clot, as well as serum. Samples should be frozen at -20°C, however -80°C is preferred for some testing, such as electrophoresis. A drop of whole blood can also be stored on an FTA card for future molecular testing, including genetics. Future research of clinical cases could also include survivors and non-survivors, to further examine ante-mortem findings in the different groups; which are potential prognostic indicators (Stacy et al. 2013). Released rehabilitation turtles can also be fitted with satellite tags to determine the success rate of rehabilitation (Seaturtle.org 2018b, Robinson et al. 2021); however, funding for satellite tags for these rehabilitation cases is often limited, with external funding required.

The number of sea turtles stranding across WA is still largely unknown; with most turtles recovered from populated areas, and areas which are patrolled. Ideally all stranded turtles in WA would be reported; however, this is unlikely, given the remoteness of parts of the state. Standardised beach surveys could serve as an alternative and consistent way to monitor sea turtle strandings. By recording stranded turtle monitoring effort, changes to sea turtle strandings over time may be observed (Stacy et al. 2017b).

Other data could also be collected from sea turtles in WA, which are not specifically health-related data but could still be useful for future research. For example, there are currently no standard morphometrics, such as CCL or CCW, for sea turtles in WA; and these could assist with body condition scoring. Genetic studies could also be included in health and disease research. Genetic analyses would help to develop a clearer picture about disease distribution and populations affected, as well as foraging

grounds for nesting turtles and breeding/nesting areas for foraging turtles. It would also be worthwhile to undertake flatback gastrointestinal content analysis of samples collected at necropsy, as the diet of flatback turtles for all life-stages remains largely unknown.

6.3.5 Collaborations

Collaborative projects are generally more successful than those undertaken in isolation, as more can be achieved by working together (Ryser-Degiorgis 2013). Indeed, in the field of wildlife health, establishing effective research collaborations are critical to achieving successful outcomes. In this project, numerous interagency collaborations have been built and demonstrated. Collaborations with universities provided access to specialists such as wildlife veterinarians, veterinary pathologists, clinical pathologists, reptilian diagnostic specialists, parasitologists and other marine research scientists. Perth Zoo and other veterinarians provided health advice, and access to live animals for ante-mortem diagnostics and dead animals for fresh necropsies. Rehabilitators and wildlife carers provided care for live animals and access to live animals for ante-mortem diagnostics, as well as dead animals for fresh necropsies. The lab at DPIRD assisted with diagnostic services such as microbiology and electron microscopy. Indigenous ranger groups and other marine rangers working in the field were usually the first to respond, report and recover stranded turtles. With regards to the fisheries industry and the large-scale resources industry, continued development of collaborations would improve conservation outcomes. For example, the fisheries industry could provide additional information about turtle and fisheries interactions. In industrial and development areas, turtles are more at risk of interaction with heavy machinery, such as dredging equipment, as such environmental officers should receive training in injured turtle rescue, stranded turtle reporting as well as retrieval and storage of cadavers for research on the advice of DBCA. Finally, it is imperative research findings are communicated back to research partners and all relevant stakeholders, to ensure the continuation of collaborative partnerships (Ryser-Degiorgis 2013).

The high numbers of turtles reported and rescued by members of the public shows the importance of the public in relation to sea turtle conservation. Increasing public awareness through improved communication campaigns and community involvement could improve stranded turtle reporting rates. There are also other ways in which collaborations with the public could improve stranded turtle reporting, such as citizen science applications (Earp and Liconti 2020).

6.4 Conclusions

Through health assessments and disease investigations, this thesis established current baselines for health and disease of sea turtles in WA. These baselines are essential and provide a number of crucial functions, which include serving as a reference point for future studies to monitor changes in population health and disease levels, detecting emerging diseases and identifing disease threats to populations. Furthermore, the health of an individual member of the broader population, is pivotal to their survival and ability to thrive and perform their role in the ecosystem. Sea turtles play a critical function in the ecosystem (Pritchard 1997), frequently referred to as sentinels of environmental health (Aguirre and Lutz 2004); and likewise the environment has a strong influence on the health and normal function of the turtle. For example, environmental factors such as poor water quality may result in suboptimal health and subclinical disease, hindering the ability of the sea turtle to reproduce and survive. Flint et al. (2017) found other environmental factors such as extreme weather events, for example flooding, were correlated with increased marine turtle stranding incidences seven to 12 months later, with reduced lag time post-flooding for stranding in embayments. When considering the One Health approach, where the health of humans, animals and the environment are inter-connected (Daszak et al. 2000), the ill-health or removal of sea turtles from the ecosystem may indicate an unhealthy environment; and clearly, maintaining a healthy environment is also in the best interest for humans.

This thesis contributes to the global knowledge base of health and disease of sea turtles and fills regional knowledge gaps through research of sea turtles in the east Indian Ocean. On a global scale, this research has detected previously unreported diseases in flatback turtles, including a novel malarial disease, and established the first flatback turtle blood RIs, all of which will have major conservation implications for ongoing species management. Clinically, blood RIs can be used for flatback turtles in rehabilitation for disease diagnosis, monitoring progress and assessing prognosis. From a research perspective, these flatback RIs can assess current health status and serve as a baseline for which future studies can be compared to monitor population health over time. On a regional scale, diseases found in our study can be used to understand disease threats to specific regions or populations. Our research also contributes to local, state and national levels, with the potential for information gained during this project to be used in future sea turtle recovery plans when assessing the impacts of infectious and non-infectious disease threats, marine debris interactions, fisheries interactions, and vessel strikes on WA and Australian sea turtles (Commonwealth of Australia 2017b).

Sea turtles face numerous different threats as they migrate between ecosystems throughout their complex and varied life cycles (Lutcavage et al. 1997). Globally, while many threats to sea turtles are anthropogenic either indirectly and directly, natural disease threats may also play a significant role in morbidity and mortality (Witherington 2017). With emerging marine diseases on the rise (Ward and Lafferty 2004), and the ever-increasing impacts of humans on sea turtle health and survival, there is an urgency to improving our understanding of sea turtle health and disease. The health and disease baselines developed in this project have built the foundation for the health status of sea turtles in WA, and serve as a platform to further develop health and disease research projects in areas where knowledge gaps have been identified and where further research is required. Considering how little is known about sea turtle health and disease in WA, research into health and disease should be continued because of the central role of health in the conservation and survival of sea turtles. Adequate funding and resources are required for managers of sea turtles in

WA; and should also be factored into future health and disease research plans for the state. Given their migratory nature and lack of barriers in the ocean, managing turtles requires global cooperation. Through global cooperation and local action, we can contribute to the assessment and management of sea turtle health and disease, and ensure the long-term survival of sea turtles into the future.

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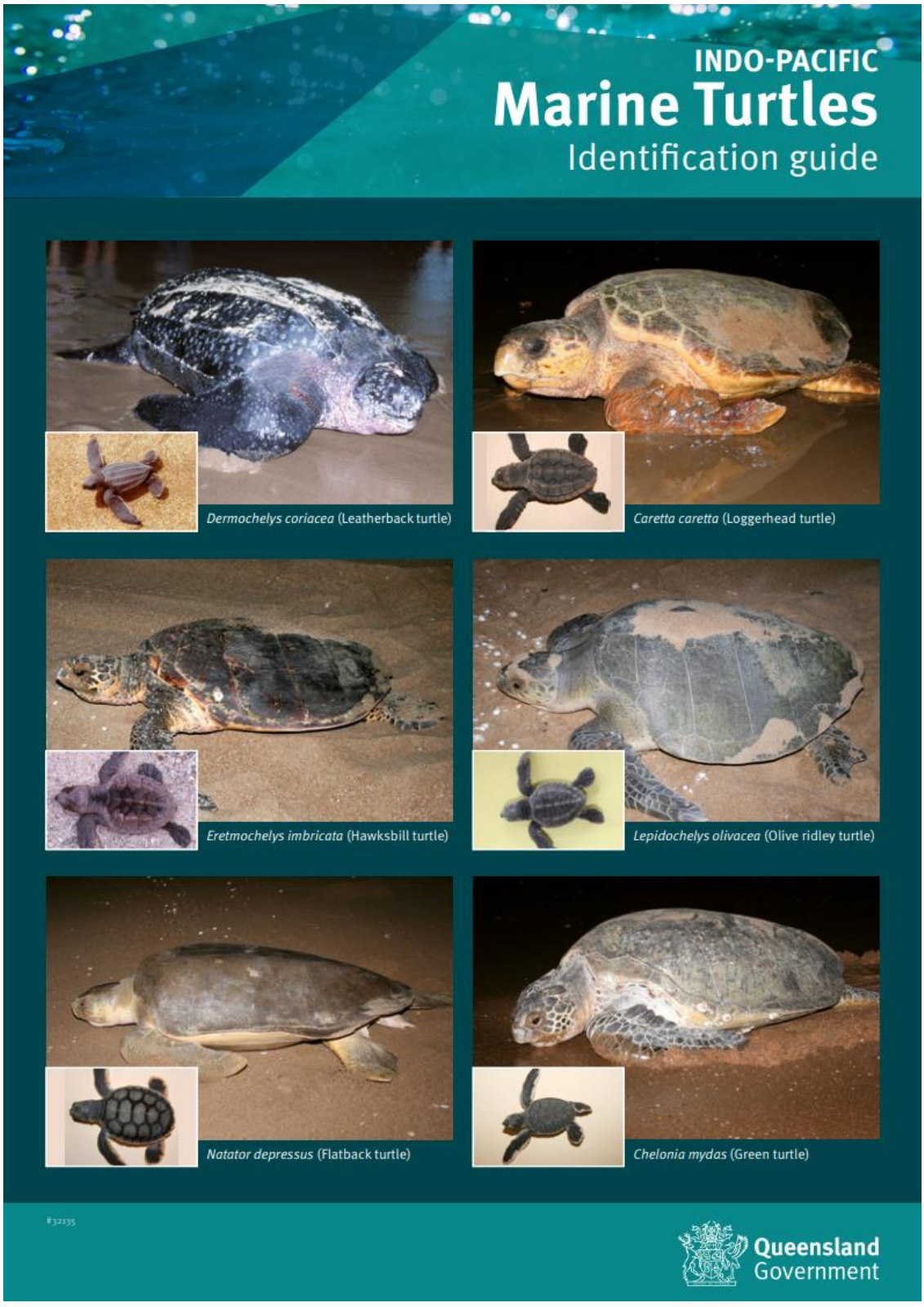
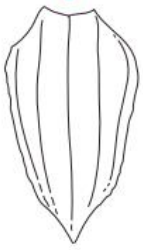
APPENDIX 1: Indo-Pacific marine turtle identification guide.



Identification key

Carapace with
- 5 distinct ridges
- no large scales
- no claws

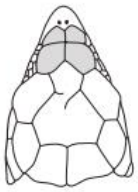


Dermochelys coriacea
(Leatherback turtle)

Carapace with
- no continuous ridges
- large scales
- claws on flippers

4 pair costal scales

2 pair prefrontal scales
- thick overlapping carapace scales



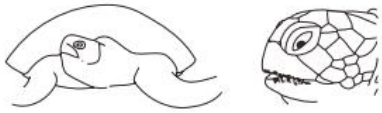
Eretmochelys imbricata
(Hawksbill turtle)

1 pair prefrontal scales
- no thick overlapping carapace scales

- carapace low domed with upturned edges
- preoccular scales
- colour olive grey

Natator depressus
(Flatback turtle)

- carapace high domed
- no preoccular scales
- colour light to dark green with dark mottling

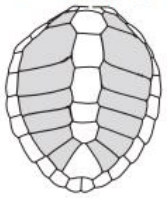


Chelonia mydas
(Green turtle)

5 pair (rarely 6) costal scales
- carapace longer than wide
- colour red-brown to brown
- no pores in scales of bridge

Caretta caretta
(Loggerhead turtle)

6 pair or more costal scales
- carapace approximately circular
- colour grey green
- pores in scales of bridge



Lepidochelys olivacea
(Olive ridley turtle)

APPENDIX 2: DBCA marine turtle stranding form.

WA MARINE TURTLE STRANDING FORM

Please record the following information for **ALL** sick, injured or dead stranded marine turtles in WA.
Please send form and photos to Department of Biodiversity Conservation and Attractions, Marine Science Program
Address: Locked Bag 104, Bentley Delivery Centre, 6983 or Email: turtles@dbca.wa.gov.au

DATE: _____ **(DD/MM/YYYY)** **TIME:** _____ **(24 hour)**

FOUND BY: _____ **PHONE NUMBER:** _____

EMAIL ADDRESS: _____

LOCATION: _____

Latitude: _____ . _____ °S **Longitude:** _____ . _____ °E

STATUS: ☐ Alive **Condition/Behaviour:** _____

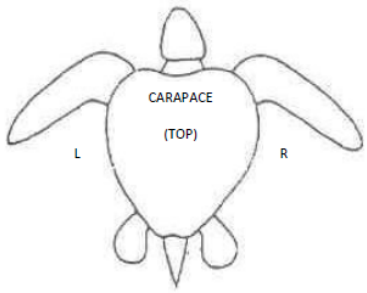
☐ Dead **Select code from options below to best describe state of carcass**

☐ (D1) Live but subsequently died
☐ (D2) Carcass in good condition (fresh/edible)
☐ (D3) Carcass fair (decomposed but organs intact)
☐ (D4) Carcass poor (advanced decomposition)
☐ (D5) Mummified carcass (skin holding bones)
☐ (D6) Disarticulated bones (no soft tissue remaining)

PHOTOGRAPHS: are essential for identification and assessment. Please take the following photos:-
☐ Carapace (top shell) ☐ Head (front and above) ☐ Any abnormalities ☐ Turtle in-situ **(see overleaf for tips)**

SPECIES (see key overleaf):
☐ Green
☐ Loggerhead
☐ Flatback
☐ Hawksbill
☐ Olive Ridley
☐ Leatherback
☐ Unknown

DISTINGUISHING FEATURES: (indicate on diagram)
☐ Obvious damage/injuries
☐ Missing limbs
☐ Tumours/growths
☐ Barnacles
☐ Algal growth on carapace
☐ Tagging scars



EXISTING TAG NUMBERS:
Left flipper _____
Right flipper _____

NEW TAG NUMBERS:
Left flipper _____
Right flipper _____

MEASUREMENTS:

Curved Carapace Length (CCL): _____ cm	☐ Measured	☐ Estimated
Curved Carapace Width (CCW): _____ cm	☐ Measured	☐ Estimated
Tail Tip to Carapace: _____ cm	☐ Measured	☐ Estimated
Maximum Head Width (HW): _____ cm	☐ Measured	☐ Estimated

SEX*: ☐ Male ☐ Female ☐ Unknown

MATURITY*: ☐ Hatchling ☐ Juvenile ☐ Adult ☐ Unknown **(* if unsure, please tick "Unknown")**

FINAL OUTCOME: _____

NOTES: _____

CONTACT DETAILS (if different to above): _____

Appendix 3: Example of baseline health field datasheet (v3 updated April 2019).

WAYARTI - WA Sea Turtle Health Project
BLOOD SAMPLING FIELD FORM

Recorded by: _____

WTR # _____

Ref.Day/Ngt _____

DATE: _____ **Species:** _____

TIME: _____ **Sex:** ☐ F ☐ M ☐ U

Location: _____ **Maturity:** ☐ Immature ☐ Mature ☐ Unknown

Lat: _____ **Long:** _____

ACTIVITY @ Capture
On Land: ☐ Emerging ☐ Body pitting ☐ Egg chambering ☐ Egg laying ☐ Covering ☐ Returning
In water: ☐ Swimming ☐ Diving ☐ Foraging ☐ Resting ☐ Lethargic ☐ Unknown
☐ Other _____ # capture attempts _____

Catch method: ☐ Hand ☐ Net **Did the turtle lay eggs?** ☐ Y ☐ N ☐ Unknown

Weather/ Ocean Conds Air temp _____ °C **Clouds** ☐ clear ☐ low fog ☐ Patchy cloud ☐ high overcast _____

SST: _____ °C **Sea State** (Beaufort) _____ **Water depth:** _____ m _____

JELLIES: Type _____ **Density** ☐ low ☐ med ☐ high **Notes:** _____

MEASUREMENTS Temperature: Internal _____ °C External _____ °C

Weight: _____ kg ☐ Actual ☐ Estimate

CCL: _____ cm CCW: _____ cm MALES: CT: _____ cm VT: _____ cm PT: _____ cm

IDENTIFICATION

Existing Tag(s) ☐ N ☐ Y Existing microchip ☐ N ☐ Y

New/Existing Tag L: _____ New/Existing Microchip: _____

New/Existing Tag R: _____ PUT STICKER HERE

Biospy ID: Other ID/Notes:

BASIC PHYSICAL EXAMINATION (SEE DIAGRAM ON REVERSE)

Body Condition	☐ Good	☐ Average	☐ Poor	☐ Emaciated	BCS: /5
Eyes	☐ NAD	☐ NE	☐ Abnormal:		
Oral cavity	☐ NAD	☐ NE	☐ Abnormal:		
Cloaca	☐ NAD	☐ NE	☐ Abnormal:		
Carapace	☐ NAD	☐ NE	☐ Abnormal:		
Plastron	☐ NAD	☐ NE	☐ Abnormal:		
Skin: Head/Neck	☐ NAD	☐ NE	☐ Abnormal:		
Flippers	☐ NAD	☐ NE	☐ Abnormal:		
Tail	☐ NAD	☐ NE	☐ Abnormal:		
Any evidence of FP/scarring?	☐ N	☐ Y:			
Barnacles	☐ N	☐ Y:			

BASIC MUSCULOSKELETAL EXAM

☐ NAD ☐ NE ☐ Abnormal: _____

BASIC NEUROLOGICAL EXAM

☐ NAD ☐ NE ☐ Abnormal: _____

Notes:

BLOOD SAMPLES Collected by: _____ Time restrained prior to collection: _____ mins

Collection Collected ☐ First ☐ After: _____

Date: _____ Restraint method: _____

Start time: _____ **EDNA**

Sample site ☐ Dorsocervical sinus Cloacal swab ☐ Y ☐ N

Method ☐ 18g needle & syringe Reference ID: _____

Volume: _____ ml **Heavy metals**

Finish time: _____ 2x Skin biopsy ☐ Y ☐ N

Attempts: _____ Shell scraping ☐ Y ☐ N

Stable isotope blood ☐ Y ☐ N

TIME OF RELEASE: **ACTIVITY @ Release:**

Updated 10/04/2022

WTR #

BLOOD SAMPLES Temperature time: _____ Blood collection to temp _____ mins

Abaxis CG4+ Results: (Ref) _____ Time: _____ Blood collection to testing _____ mins

Lactate _____ pH _____ pCO_2 _____ pO_2

TCO_2 _____ HCO_3 _____ BE _____ sO_2 _____

Intitial handling

Refrigeration (instant) ☐ Y ☐ N Time: _____

Centrifugation (<6hr) ☐ Y ☐ N Time: _____

In field procedures

PCV: _____ ☐ Fresh ☐ Li Hep Tubes: ☐ Plain ☐ Hep.

TP: _____ g/l Plasma: _____

Smear ☐ Fresh ☐ LiHep

FTA ☐ Y ☐ N

Final handling/Distribution

Vet Path (4ml)

Smear ☐ Fresh ☐ LiHep

Blood ☐ 1ml plasma ☐ Whole blood LiHep ☐ Fluoride/Oxalate

Time & Date analysed: _____

Heavy metals (1ml)

blood ☐ 1ml Whole blood LiHep

Toxicology (3ml)

blood ☐ 1ml Whole blood LiHep ☐ 1ml Plasma LiHep ☐ 5-6ml whole blood banked

Frozen (kept) ☐ N ☐ Y Time: _____

Sample type: ☐ Plasma Vol: _____ ml No. vials: _____

☐ Clot Vol: _____ ml No. vials: _____

☐ Whole blood Vol: _____ ml No. vials: _____

OTHER SAMPLES: _____

Other notes:

KEY TAKE PHOTOS OF ANY ABNORMALITIES

1. Injury or Lesion

A Abrasion (scrape e.g. skeg)
B Bruise (haematoma)
C Constriction (Ligation-note discolouration)
D Deformity
E Epidermal changes (scute,skin)
F Fracture
G Growths (tumours eg FP)
H Haemorrhage (bleeding)
L Luxation (dislocation)
M Missing (amputation -note shape and %)
P Puncture/perforation
T Tear (laceration e.g. prop)
U Ulcer (deep open infected wound)

2. Severity

1 Minor
2 Moderate
3 Severe

3. Stage of healing

I Fresh
II Partially healed
III Healed

4. Epibiota est. % cover type/colour

// Algae (shade) _____ _____
minimal light mod heavy Growth

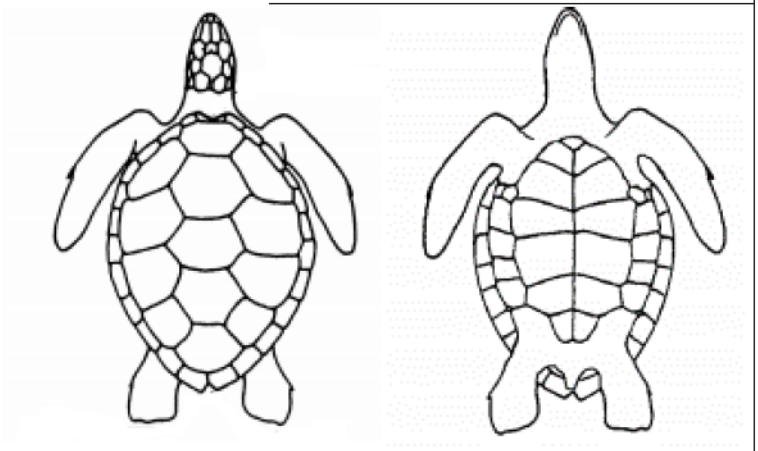
O Barnacle (circle) _____
size Xs <0.5; S <1; M 1-2.5; L 2.5-5; XL >5cm
density 0 <10 10-20 21-30 >30 (pls circle both)

* Other (star, species)

5. Causes

☐ Traumatic e.g. Predator, conspecific, boat or fishery interaction, entanglement, other
☐ Infectious e.g. bacteria, fungi, virus (FP), parasite, epibionts,
☐ Other E.g. Neoplastic, Developmental, Vascular, Environmental, Degenerative, Metabolic, Iatrogenic
☐ Unknown _____
☐ Injured during catch up

6. Pictures ☐ _____



Updated 10/04/2022

Appendix 4: Perth Zoo Sea Turtle Admission Form.

DPaW – MARINE TURTLE ADMISSION FORM

PERTH ZOO

SPECIES: _____ **ID No:** _____ **Enclosure:**_____

AGE: _____ **SEX:** _____ **DATE:** _____

RESCUER/WILDLIFE OFFICERS NAME: _____

CONTACT NUMBER (am/pm) _____ Mobile: _____

OFFICE USE ONLY

Handover sheet stapled to admission sheet	☐	Fate added to Wildlife Book	☐	Costing completed	☐ 1st	☐ revisit ☐ revisit

HISTORY

(Donor / Rescuer to complete)

DATE FOUND: _____

EXACT LOCATION ANIMAL WAS FOUND (include details)_____

_____

WHAT WAS THE ANIMAL DOING WHEN FOUND? Ie: in water, on dunes etc)? _____

_____

WHAT IS WRONG WITH THE ANIMAL (any obvious injuries)? _____

_____

HAS THE ANIMAL BEEN SEEN BY A VET, IF SO WHICH ONE: _____

_____

HAS ANY MEDICAL TREATMENT BEEN GIVEN, IF SO WHAT? _____

_____

HAS THE ANIMAL BEEN FED? If yes – What / How Much/ When:_____

_____

HOW HAS THE ANIMAL BEEN HOUSED? (e.g. fresh/salt water, temperature)

MEASUREMENTS:

Curved carapace length (CCL) _____ cm

Curved carapace width (CCW) _____ cm

Tail length (from carapace) _____ cm

Maximum head width _____ cm

DPaW notified of admission ☐ Date: _____

Discharge emailed to Cameron@DPaw ☐

E:_Admin-Franklin\Master Sheets Hospital Vet Dept\Dpaw & Fauna\Dpaw Marine Turtle Admission Form.Doc

Appendix 5: Sea turtle necropsy datasheet.

SEA TURTLE NECROPSY DATASHEET

WT # | Accession Date: | MU Path. #

Rsch no.: | Date of death: | Necropsy loc.:

Necropsy Date: | Time: | Vet: | Scribe:

ID

Species C.mydas ☐ C.caretta ☐ N.depressus ☐ E.imbricata ☐ L.olivacea ☐ D.coriacea ☐

Age class Ha ☐ Post-Ha ☐ Juv ☐ Sub-Ad ☐ Ad ☐ Sex M ☐ F ☐ U ☐

Microchip Y ☐ N ☐ NC ☐ No. Other ID

Morpho-metrics

Weight	Measurements (cm)										
Kg	CCL	CCW	SCL	SCW	SD	ND	HL	HW	PL	CT	VT	PT

Act. ☐ Est. ☐ Notes

Scute pattern

Central			Costal		Marginal		Pre-frontal		Post-parietal			Pre-ocular		Gular			Infra-marginal	
pre	mid	post	L	R	L	R	L	R	L	mid	R	L	R	L	mid	R	L	R

Notes

History (Date/Loc found, clinical signs, treatment etc)

Cadaver condition Fresh ☐ Frozen/Thawed ☐ Refridgerated ☐ hrs

Manner of Death Spontaneous ☐ Euthanasia ☐ Agent & Route:

Body Condition Good ☐ Average ☐ Poor ☐ Emaciated ☐

Body Decomposition Score D1 ☐ D2 ☐ D3 ☐ D4 ☐ D5 ☐ D6 ☐

External (including skin, plastron, carapace, eyes, cloaca, epibiota)

Updated 12/11/2018

SEA TURTLE NECROPSY DATASHEET

Coelmic cavity (peritoneum, fluid, fat reserves, mesentery)		*CC fluid* _____ *Fat status* _____ *Musc mass* _____
Respiratory system (nasal cavity, trachea, bronchi, lungs)		
Cardiovascular system (heart, pericardium, great vessels)	*Heart wt (g)* *Heart meas. (cm)*	_____ _____
Alimentary system (oral cav., oes., crop, stomach, int., caecum, liver, G.B., panc.)	*Liver wt (g)* *Liver meas. (cm)*	_____ _____
GIT contents (food, foreign bodies, parasites)	*Stomach contents wt (g)* *Overall gutfill %*	_____ _____

Updated 12/11/2018

SEA TURTLE NECROPSY DATASHEET

WT # **Nec date:** **Path #**

Urogenital system

(kid., blad., ureters, urethra, gonads, oviduct, epi, penis, gravid?)

Gonad wt(g)L/R

Gonad meas. L/R

Kidney wt(g)L/R

Kidney meas. L/R

Haematopoeic system (thymus, spleen, bone marrow)

Spleen wt(g)

Spleen meas. (cm)

Endocrine system (thyroid, adrenals, salt gland)

Adrenal wt (g) L/R

Adrenal meas.L/R

Thyroid wt (g)

Thyroid meas.

Nervous system (brain, spinal cord, meninges)

Brain wt (g)

Brain meas.

Musculoskeletal system (muscle, bone, joint, ear)

Parasitology

	cc	lu-tr	he-ve	spl	liv	kid	br	sg	gb	blad	oe-cr	stom	SI	LI			
Org wash																	
Parasites																	

Notes:

Faeces present: Y / N Faecal float: Y / N Results/Notes:

Summary PM findings, preliminary gross morphological diagnosis

Photos	Body		Head			Flippers	Abnorm-alities	Internal		Parasites	ID marks	FBs
	DV	VD	DV	RoCd	Bilat			Organs in-situ	GIT			

Updated 12/11/2018

SEA TURTLE NECROPSY DATASHEET

	Code 1 - Standard PM	Bio-metric	Histo Std	Histo Add	Parasite	Culture	Frozen	Heavy metals	Other toxicol	Life hist.	Sample description, Where/When sent
Respir-atory	Nasal cavity			☐	☐		☐				
	Trachea			☐	☐		☐				
	Lung		☐			☐	☐				
CVS	Heart	☐	☐		☐		☐				
	Great vessels		☐				☐				
Haemo-poietic	Spleen	☐	☐		☐	☐	☐				
	Blood						☐	☐		☐	
Digestive	Oral cav./tongue			☐							
	Oes./crop		☐		☐						
	Coelmic cavity fluid				☐		☐				
	Liver	☐	☐		☐	☐	☐	☐	☐		
	Gall bladder		☐		☐						
	Bile						☐				
	Stomach		☐		☐						
	GIT contents				☐				☐	☐	
	Pancreas		☐				☐				
	Intestine (small)		☐		☐						
	Intestine (large)		☐		☐						
	Mesentery		☐		☐						
	Cloaca			☐							
	Faeces				☐						
Uro-genital	Kidney	☐	☐		☐	☐	☐	☐	☐		
	Urinary bladder		☐		☐						
	Urine/urates						☐				
	Gonads (Repro)	☐	☐		☐				☐	☐	
Neuro-logical	Brain (CSF)	☐	☐		☐		☐		☐		
	Spinal cord (SCF)		☐				☐				
	Ganglion						☐				
	Eyes			☐							
Endocrine	Adrenals	☐	☐		☐		☐				
	Thyroid		☐		☐		☐				
	Thymus			☐							
	Salt gland		☐		☐		☐				
Other	Shell			☐	☐			☐			
	Skin			☐	☐		☐	☐		☐	
	Bone/joint/marrow			☐				☐		☐	RIs
	Muscle			☐				☐	☐		
	Fat			☐				☐	☐		
Misc.	Other lesions										
No. pots/samples		—			/						

Updated 12/11/2018

Appendix 6: Necropsy checklist.

NECROPSY CHECKLIST

Date Checked:_____

Pre/mid/Post PM no:_____

✓x	Qty missing	Qty	Item	Notes
			SMALL FIELD KIT	
			Upper tray	
			Pens/Markers/Pencils/Paint marker	
		10	Tongue depressors	
		3	Laminated lables	
		1	Measuring tape	
		1	Flexible tape	
		3 boxes	Scalpels (10,20,22)	
		1	Stone	
			Bottom Section	
			Instruments	
		3	Butcher Knieves	
		3	Forceps	1 in SMALL NECROPSY KIT
		6	Scissors	1 in SMALL NECROPSY KIT
		2	Haemostats	
		2	Clamps	
		3	Scalpel holder	1 in SMALL NECROPSY KIT
		1	Spatula	
		2	Probes (Sharp and blunt)	
		1	Bone cutters	
		1	Bone Saw	
		1	Flathead screw driver	
		1	Poultry Shears	
			Other	
		2	Metal rulers (large and small)	Move b/w kits. White murdoch ruler
		1	Steel	
		1	Calipers	
		1	Kitchen scales	
			LARGE FIELD KIT	
			Upper tray	
		2	Scrubbing brush (instrument & nails)	sponge
		1	Washing up gloves	
		1 pckt	Wetwipes	
		1 roll	Paper towel	and in parasit box
		10	Aprons	non-disposable
		1 roll	Bin bags	
		1 ball	String	
		1	Blow torch	
			Bottom Section	
		1	long handled segateurs	parasite container
		1 box	Gloves	
		1	Coveralls	
		1	Safety glasses	
		10	Masks (surgical)	
		5	Masks (P2)	
		1	Measuring jug	
			Syringes	
			Needles	
		1 box	Slides	
		6	Slide holders	
		1 box	Coverslips	
		15	Swabs (Gel and dry and charcoal)	Stuarts medium
		6	FTA cards	
		3	Body bags	
		roll	Garbage bags	
		cont.	Cable ties	
		1	Gas bottle	
		1	Sharps container	

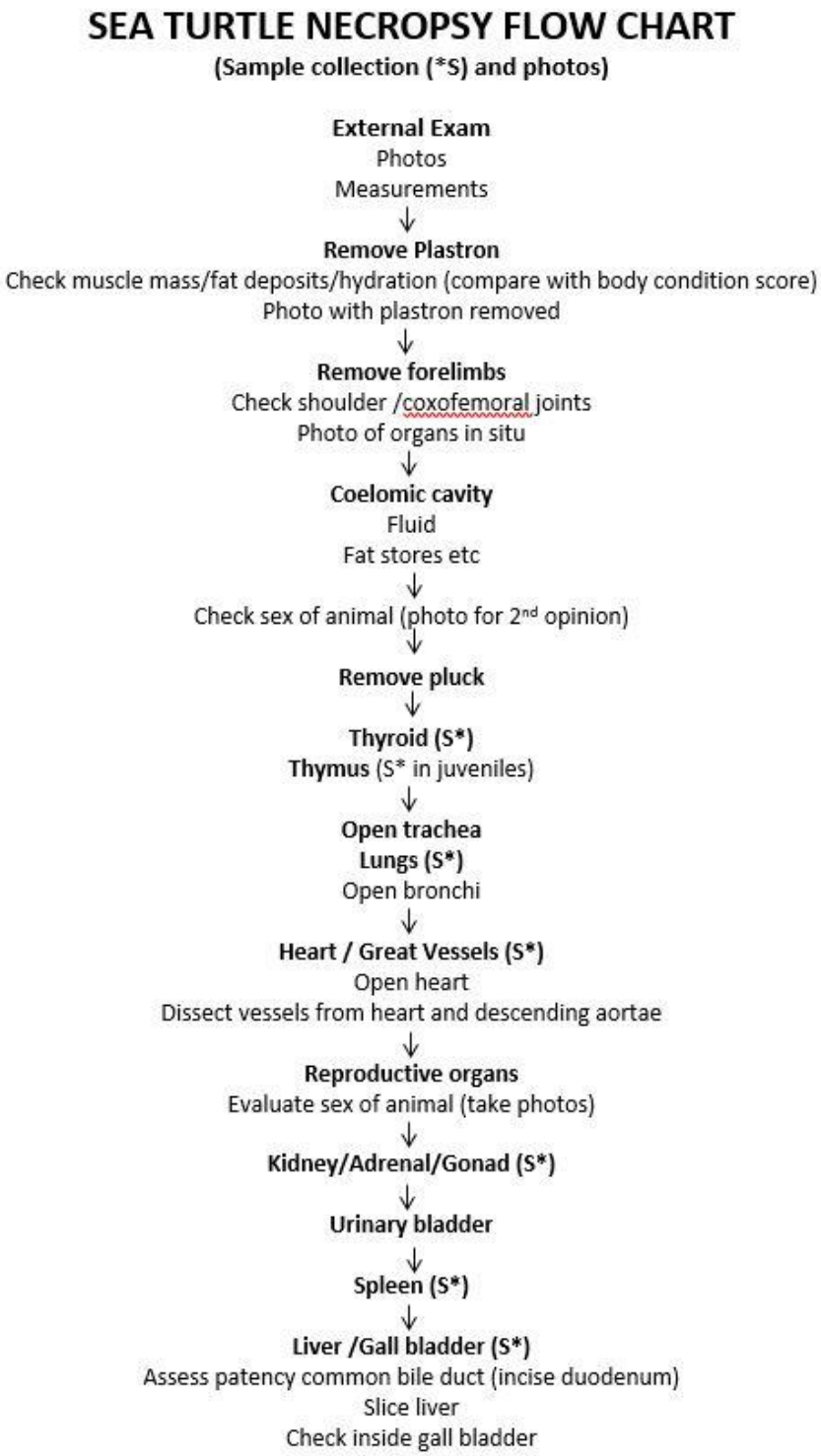
NECROPSY CHECKLIST

Date Checked:_____

Pre/mid/Post PM no:_____

✓ x	Qty missing	Qty	Item	Notes
			GREEN CRATE (Disposables)	
		6	1L specimen pots	
		10	250ml specimen pots	
		40	70ml specimen pots	
		50	yellow top vials	
			Glass jars (variety of sizes)	
		10	Weigh trays (Large and small)	
			Zip lock bags (variety of sizes)	
		roll	Aluminium foil	
			Dry swabs	
			WHITE CRATE (Parasitology and Boards etc)	
		2	Cutting board (large and small)	
		1	Blueboard	
		2	Trays	
		30	Pasteur pipettes	
		15	Petri dishes (small and large)	
		2	Buckets	
		1	NaCl	8g/L; Fill container up from bag
*		1	90% ethanol	
		1	10% NB formalin	
		1	PBS	20ml distilled water to 1ml PBS concentrate
		1	vaseline	
			Waterproof paper and pencils	Lables for eth vials - put in separate bag
			Used pots in foam eski	
		1	Faecal float soln	
		2	Faecalyzer	
			Datasheets, Guides etc	
		10	PM Datasheets	
		1	Checklist	
		1	Copy of latest PM list	
		5	Pre-printed lables (variety of sizes)	Need L7651 lables
		5	Lab submission forms	
		10	Volunteer forms	
		1	Ring binder folder	With above forms and others e.g. stranding forms
		1	Plastic envelop folder	PM & ID guides, licences and other info sheets
		1	Sea Turtle Anatomy	
		2	Clipboards	
			Necropsy tags	
			OTHER IMPORTANT ITEMS TO BRING	
			Freezer	
		2	Icebricks	
			Store room	
		1	Eski	
		1	Stereomicroscope	
		1	Head torch	move b/w kits
*		1	Microchip Scanner + spare 9V batt	move b/w kits
			Office	
*			Camera + batt + charger + SIM card	charge camera, sim in!
			Other items to maybe bring	
		1	Refractometer	
		1	Pliers	
		1	Tape	
		1	Stapler	
		1	Spray paint	
		1	Innox	
		1	Stanley knife	
		1	200kg scales	
		1	Engel	
		1	Wirecutters	
			Newspaper	
		1	Spray bottle with cetrigen	

Appendix 7: Necropsy flow chart



REMOVE Alimentary tract AND PUT ASIDE

↓

Head incision (caudal to eye)

↓

Brain

Include spinal cord sample, frontal lobe for frozen sample

Salt glands

↓

Eye

↓

Return to GIT

Pancreas/SI (S*)

Tongue/Oesophagus/Crop (Crop only in some green turtles)

Stomach/SI/LI (S*)

Gut fill, contents, foreign bodies

Open sections, care with handling, mucosal layer easily damaged

Additional optional samples in POT 2: Bone/Joint/Bone marrow/Shell, Skin, Muscle, Nasal cavity, Trachea, Oral cavity/Tongue, Cloaca Fat Eyes

Appendix 8: Measurement method for Beckman Coulter AU680 and Cell-Dyn 3700 automated machines using Beckman coulter/Olympus reagents unless otherwise indicated.

Analyte	Measurement Methodology
PCV	Manual haematocrit
Hb	Hemiglobin-hydroylamine, corrected RBC resistant mode, photometric (Sysmex uses SLS-HGB)
RBC	Electrical impedance
WBC	Manual smear
MCV, MCH, MCHC	Calculations
Na	Indirect ion selective electrode
K	Indirect ion selective electrode
Cl	Indirect ion selective electrode
CK	Kinetic UV, NAC inactivated
AST	Kinetic UV without P5P
ALT	Kinetic UV without P5P
ALP	Kinetic colorimetric with AMP, PNPP
Total protein	Photometric biuret
Albumin	Bromocresol green dye-binding
Globulin	Calculation
Urea	Kinetic urease UV
Uric acid	Uricase colorimetric
Triglyceride	Enzymatic
Cholesterol	Enzymatic colorimetric CE, CHOD, POD
Ca	Photometric colorimetric Arsenazo III
P	Photometric phosphomolybdate UV
Bilirubin	Photometric colour diazotization DPD
Glucose	Enzymatic UV hexokinase
Magnesium	Photometric xylidyl blue
Fe	Photometric colorimetric TPTZ (AU680)
LDH	Kinetic UV lactate to pyruvate (Advia)
Bile acids	Enzymatic colorimetric (Randox reagents, AU680)
GLDH	(Randox reagents, AU680)
In house*	
PCV	Manual haematocrit
TPS	Refractometer
Na	I-stat direct ion-selective electrode
K	I-stat direct ion-selective electrode
Cl	I-stat direct ion-selective electrode
Urea	I-stat urease, NH3, ion-selective electrode
Glucose	I-stat oxidation, ion-selective electrode
HCT	I-stat conductivity

* Only methods for reported analytes were included

Appendix 9: p-values for 50* analytes using the appropriate parametric and non-parametric methods to test the difference between source population (nesting and foraging) (post-global exclusion criteria and pre-outlier removal) (bolded values are not statistically significant).**

Analyte***	n	Mann-Whitney U test	t-test	Statistically significant group direction
PCV	100	0.004		Foraging ↑ / Nesting ↓
Hb	113	0.008		Foraging ↓ / Nesting ↑
RBC	111	<0.001		Foraging ↑ / Nesting ↓
MCHC	113	<0.001		Foraging ↓/ Nesting ↑
MCH	111	<0.001		Foraging ↓ / Nesting ↑
MCV	111	<0.001		Foraging ↓/ Nesting ↑
WBC	122	0.035		Foraging ↑ / Nesting ↓
Heterophils (differential/absolute)	127/122	<0.001/<0.001		Foraging ↓ / Nesting ↑
Lymphocytes (differential/absolute)	127/122	<0.001 (absolute)	<0.001 (differential)	Foraging ↑ / Nesting ↓
Monocytes (differential/absolute)	127/122	<0.001/<0.001		Foraging ↑ / Nesting ↓
Eosinophils (differential/absolute)	127/122	**0.231** (absolute)	**0.067** (differential)	
Basophils (differential/absolute)	127/122	<0.001/<0.001		Foraging ↑ / Nesting ↓
Heterophil: Lymphocyte ratio	104	<0.001		Foraging ↓ / Nesting ↑
CK	124	0.005		Foraging ↑ / Nesting ↓
AST	124	0.025		Foraging ↑ / Nesting ↓
ALT	124	0.011		Foraging ↑ / Nesting ↓
ALP	124	<0.001		Foraging ↑ / Nesting ↓
Bilirubin T	124	0.006		Foraging ↓ / Nesting ↑
BUN	124	<0.001		Foraging ↑ / Nesting ↓
Bile acids	124	**0.064**		
Uric acid	124	<0.001		Foraging ↑ / Nesting ↓
Glucose (fluoride oxalate)	74	<0.001		Foraging ↓ / Nesting ↑
Cholesterol	124	<0.001		Foraging ↓ / Nesting ↑
Triglyceride	124	0.001		Foraging ↓ / Nesting ↑
Na	124	**0.109**		
K	124		<0.001	Foraging ↓ / Nesting ↑
Cl	124		<0.001	Foraging ↑ / Nesting ↓
Total Protein	124		**0.423**	
Albumin	124	**0.244**		
Globulin	124		**0.595**	
Ca	124	<0.001		Foraging ↓ / Nesting ↑
P	124	<0.001		Foraging ↓ / Nesting ↑
Ca:P	124	0.018		Foraging ↑ / Nesting ↓
Mg	124	<0.001		Foraging ↓ / Nesting ↑
Fe	124	**0.051**		
LDH	124	0.021		Foraging ↑ / Nesting ↓
GLDH	66	**0.812**		
In-house PCV	131	0.002		Foraging ↑ / Nesting ↓
Total solids	158	**0.15**		
Chem8+ Na	73	**0.762**		
Chem8+ K	73		0.012	Foraging ↓ / Nesting ↑
Chem8+ Cl	73		0.001	Foraging ↑ / Nesting ↓
Chem8+ Glucose	72	0.003		Foraging ↓ / Nesting ↑
Chem8+ BUN	73	<0.001		Foraging ↑ / Nesting ↓
Chem8+ HCT	73		**0.224**	

* Only 50 analytes comparable for initial combined dataset (only Li-Hep glucose only in nesting turtles,

** Appendix 9 Supplementary Table differs to Table 3.6, which also examines source statistical differences as a combined dataset, by instead comparing the final nesting and foraging groups i.e. after outlier removal.

*** all WBC, differential and absolute values are estimated

Appendix 10: Outliers detected for laboratory and field nesting flatback turtle analytes and outlier effect on mean (all outliers based on untransformed data).

Analyte	No. of outliers (% of total)	Mean of outliers	Mean with outliers	Mean without outliers	No. outliers removed	Outlier values removed (animal ID)
RBC	2 (3.1)	0.48	0.24	0.24	0	
MCHC	5 (7.6)	229.7	315.1	322.1	0	
MCH	6 (9.4)	1158.3	520.9	455.0	4	1383(WTR108), 1300(WTR92), 1180(WTR41), 1127(WTR31)
MCV	5 (7.8)	3877.2	1673.2	1486.4	2	4568(WTR108), 4300(WTR92)
WBC	1 (1.1)	18.30	7.97	7.85	1	18.3(WTR29)
Heterophils (absolute)	2 (2.3)	11.07	4.43	4.28	2	11.16(WTR143), 10.98(WTR29)
Monocytes (differential)	4 (4.3)	16.0	5.3	4.8	3	17(WTR63), 15(WTR29), 15(WTR83)
Monocytes (absolute)	4 (4.6)	1.92	0.44	0.37	2	2.75(WTR29), 2.13(WTR63)
Eosinophils (differential)	2 (2.2)	33.00	13.1	12.7	2	35(WTR56), 31(WTR51)
Eosinophils (absolute)	3 (3.4)	2.97	1.03	0.96	2	3.57(WTR51), 2.69(WTR56)
Basophils (differential)	3 (3.2)	1.0	0.0	0.0	3	1(WTR29), 1(WTR47), 1(WTR49)
Basophils (absolute)	3 (3.4)	0.11	0	0	3	0.18(WTR29), 0.09(WTR47), 0.07(WTR49)
H: L ratio	13 (15.5)	26.0	5.90	2.21	13	87(WTR111), 45(WTR110), 39.5(WTR62), 26.3(WTR113), 24.7(WTR112), 22.8(WTR114), 20.3(WTR120), 15.2(WTR109), 13.7(WTR105), 12.3(WTR107), 11.7(WTR23), 11.1(WTR49), 8.9(WTR106)
CK	4 (4.4)	13780.3	1358.4	780.6	4	28895(WTR6), 19013(WTR141), 3669(WTR124), 3544(WTR129)
AST	2 (2.2)	536.0	196.9	189.2	2	600(WTR145), 472(WTR6)
ALT	8 (8.9)	24.6	7.5	5.9	5	43(WTR22), 42(WTR21), 27(WTR84), 17(WTR6), 15(WTR108)
ALP	8 (8.9)	251.0	70.2	52.5	4	762(WTR68), 344(WTR121), 247(WTR124), 222(WTR64)
Bilirubin T	4 (4.4)	9.23	3.2	2.92	0	
BUN	7 (7.8)	5.34	1.99	1.7	0	
Bile acids	4 (4.4)	5.8	1.6	1.4	3	7(WTR84), 6(WTR22), 5(WTR143)
Uric acid	5 (5.6)	0.109	0.064	0.062	1	0.118(WTR141)
Glucose Li-Hep	1 (2.0)	6.40	4.52	4.48	0	
Cholesterol	2 (2.2)	18.70	7.39	7.13	1	19.9(WTR143)
Triglyceride	2 (2.2)	20.25	7.7	7.42	1	21.7(WTR143)
K	2 (2.2)	5.40	4.71	4.7	1	7.3 (WTR6)
Ca	2 (2.2)	8.58	3.93	3.82	1	9.83(WTR143)
P	1 (1.1)	5.65	3.54	3.52	1	5.65(WTR29)
Ca: P ratio	3 (3.3)	1.75	1.12	1.1	1	1.81(WTR143)
Mg	4 (4.4)	4.67	4.19	4.17	2	0.02(WTR69), 6.55(WTR143)
LDH	9 (10)	1474.3	632.9	539.4	4	3037(WTR141), 2065(WTR6), 1271(WTR143), 1097(WTR108)
GLDH	5 (15.6)	169.9	48.2	25.7	2	290(WTR145), 178.7(WTR93)
Total solids	1 (1.0)	90.0	53.8	53.5	1	90(WTR143)
Chem8+ Na	6 (9.1)	139.8	146.6	147.3	1	141(WTR95)
Chem8+ K	1 (1.5)	5.70	4.52	4.50	0	
Chem8+ BUN	10 (15.2)	4.67	1.32	0.72	0	

Appendix 11: Outliers detected for laboratory and field foraging flatback turtle analytes and outlier effect on mean.

Analyte*	No. (%) outliers of total	Mean of outliers	Mean with outliers	Mean without outliers	No. outliers removed	Outlier values removed (animal ID)
Hb	4 (11.8)	94.25	101.38	102.33	2	124(WTR213), 87(WTR191)
WBC$^{\Delta}$	5 (13.5)	10.90	9.53	9.32	1	38.6(WTR191)
Heterophils (differential)$^{\Delta}$	1 (2.7)	14.0	31.1	31.6	0	
Heterophils (absolute)$^{\Delta}$	1(2.7)	10.42	2.80	2.59	1	10.42(WTR191)
Lymphocytes (differential)	1 (2.7)	8.0	47.4	48.5	0	
Lymphocytes (absolute)	1 (2.7)	17.37	4.71	4.35	1	17.37(WTR191)
Eosinophils (absolute)$^{\Delta}$	1 (2.7)	0.12	1.05	1.08	0	
Basophils (differential)	4 (10.8)	2.5	0.4	0.2	2	3(WTR149), 3(WTR150)
Basophils (absolute)	7 (18.9)	0.18	0.04	0.01	2	0.26(WTR149), 0.23(WTR150)
H: L ratio	4 (11.8)	5.06	1.10	0.58	1	7.1(WTR187)
AST$^{\Delta}$	2 (5.9)	555	255.5	236.8	1	996(WTR213)
ALT	2 (5.9)	42.5	9.6	7.5	1	65(WTR213)
ALP$^{\Delta}$	2 (5.9)	149.5	86.2	82.2	1	264(WTR186)
Bile acids$^{\Delta}$	2 (5.9)	4.5	1.8	1.6	0	
Glucose (FI-Ox)	3 (9.1)	3.33	2.79	2.74	2	4.4(WTR153), 4.2(WTR213)
K	1 (2.9)	2.40	3.84	3.88	0	
Albumin	1 (2.9)	20.0	13.4	13.2	0	
Globulin	1 (2.9)	48.0	31.4	30.9	0	
P	1 (2.9)	3.76	2.11	2.06	0	
GLDH$^{\Delta}$	1 (2.9)	318	40.65	32.25	1	318(WTR213)
Total solids	1 (2.6)	102.0	53.1	51.8	0	
Chem 8+ Cl	1 (14.3)	128.0	119.9	118.5	1	128(WTR153)
Chem8+ glucose	2 (28.6)	2.95	2.60	2.46	1	3.8(WTR153)

* Monocytes (absolute), BUN, cholesterol, triglyceride, total protein, Ca: P ratio outliers were not included as transformation resulted in narrower RIs without outliers and followed CLSI guidelines.

$^{\Delta}$ WBC, heterophil differential and absolute counts, eosinophil absolute counts, AST, ALP, bile acids and GLDH transformed values were selected as they were narrower (and had less outliers), following CLSI guidelines.

Appendix 12: Descriptive statistics for juvenile foraging flatback turtle haematology and biochemistry.

Analytes	SI Units	n	Mean	SD	Median	Min	Max
PCV	L/L	3	28.7	6.4	26	24	36
Hb	g/L	3	78.0	18.2	69	66	99
RBC	10^{12}/L	3	0.36	0.04	0.35	0.32	0.39
MCHC	g/L	3	271.7	5.8	275	265	275
MCH	pg	3	214.7	39.3	220	173	251
MCV	fL	3	798.6	99.9	748	734	914
WBC	10^9/L	3	12.22	1.85	13.28	10.08	13.30
Heterophils	%	3	18.7	6.7	22.0	11	23
Heterophils	10^9/L	3	2.25	0.80	2.22	1.46	3.05
Lymphocytes	%	3	70.3	8.4	66.0	65	80
Lymphocytes	10^9/L	3	8.65	2.05	8.76	6.56	10.64
Monocytes	%	3	7.0	1.0	7.0	6	8
Monocytes	10^9/L	3	0.84	0.07	0.81	0.80	0.93
Eosinophils	%	3	4.0	1.7	5.0	2	5
Eosinophils	10^9/L	3	0.48	0.20	0.50	0.27	0.66
Basophils	%	3	0.0	0.0	0.0	0	0
Basophils	10^9/L	3	0.00	0.00	0.00	0	0
H: L		3	0.27	0.12	0.34	0.14	0.35
CK	U/L	2	1760.5	132.2	1761	1667	1854
AST	U/L	2	242.5	167.6	243	124	361
ALT	U/L	2	8.0	4.2	8	5	11
ALP	U/L	2	31.5	3.5	32	29	34
Bilirubin T	µmol/L	2	1.93	1.38	1.9	1.0	2.9
BUN	mmol/L	2	22.45	0.78	22.5	21.9	23.0
Bile acids	µmol/L	2	1.0	0.0	1	1	1
Uric acid	µmol/L	2	0.100	0.033	0.099	0.075	0.122
Glucose FI-Ox	mmol/L	2	3.05	0.64	3.1	2.6	3.5
Cholesterol	mmol/L	2	3.00	0.57	3.0	2.6	5.4
Triglyceride	mmol/L	2	1.41	1.00	1.4	0.7	2.1
Na	mmol/L	2	157.0	1.4	157	156	158
K	mmol/L	2	4.35	0.49	4.4	4.0	4.7
Cl	mmol/L	2	126.0	0.0	126	126	126
Total protein	g/L	2	33.5	10.6	34	26	41
Albumin	g/L	2	9.5	3.5	10	7	12
Globulin	g/L	2	24.0	7.1	24	19	29
Ca	mmol/L	2	1.88	0.07	1.88	1.83	1.93
P	mmol/L	2	2.05	0.44	2.05	1.74	2.36
Ca: P		2	0.93	0.17	0.93	0.82	1.05
Mg	mmol/L	2	2.70	0.33	2.70	2.46	2.93
Fe	µmol/L	2	6.25	6.86	6.3	1.4	11.1
LDH	U/L	2	751.5	265.2	752	564	939
GLDH	U/L	2	12.65	6.29	12.7	8.2	17.1

Appendix 13: Descriptive statistics for juvenile foraging flatback turtle in-house blood results.

Analytes	SI Units	n	Mean/ RV*	SD	Median	Min	Max
PCV	L/L	3	29.0	4.4	27	26	34
Total solids	g/L	3	35.0	11.3	29	28	48
Na	mmol/L	1	148				
K	mmol/L	1	4.3				
Cl	mmol/L	1	123				
Glucose	mmol/L	1	2.8				
BUN	mmol/L	1	27.4				
HCT	L/L	1	27				

* Single RV for n=1

Appendix 14: Sex comparisons for foraging flatback turtles.

Analyte[1,2]	Female								Male								p	df	t	W
	n	mean	sd	median		min	-	max	n	mean	sd	median		min	-	max				
PCV	17	38.5	2.8	38	(	35	-	43	)	14	36.9	3.1	37	(	30	-	42	)	**0.235**			149
Hb	17	103.6	4.6	103	(	94	-	114	)	14	97.9	8.7	100	(	79	-	114	)	0.035			172.5
RBC	17	0.42	0.03	0.42	(	0.36	-	0.46	)	14	0.40	0.03	0.40	(	0.34	-	0.47	)	**0.185**	29	1.36	
MCHC	17	269.6	12.5	269	(	251	-	294	)	14	266	14	271	(	242	-	286	)	**0.418**	29	0.82	
MCH	17	250.2	22.0	251	(	206	-	278	)	14	250	24	256	(	206	-	292	)	**0.976**	29	-0.03	
MCV	17	929.9	64.8	935	(	833	-	1055	)	14	924	70	908	(	835	-	1079	)	**0.808**	29	0.25	
WBC	19	9.60	2.03	9.6	(	4.2	-	12.2	)	15	7.52	2.67	7.4	(	3.3	-	12.5	)	0.015	32	2.58	
Heterophils																						
Differential	19	27.32	10.01	27.0	(	14.0	-	62.0	)	15	36.47	13.11	36.0	(	22.0	-	71.0	)	0.013			70.5
Absolute	19	2.53	0.72	2.52	(	1.28	-	4.06	)	15	2.63	1.07	2.42	(	1.22	-	4.97	)	**0.733**	32	-0.34	
Lymphocytes																						
Differential	19	54.11	12.09	56.0	(	15.0	-	68.0	)	15	38.80	15.98	41.0	(	8.0	-	58.0	)	0.003			228.5
Absolute	19	5.29	1.56	5.38	(	0.63	-	7.83	)	15	3.13	2.16	2.85	(	0.66	-	7.25	)	0.002	32	3.4	
Monocytes																						
Differential	19	10.05	5.93	8.0	(	2.0	-	23.0	)	15	12.13	4.79	13.0	(	3.0	-	18.0	)	**0.278**	32	-1.1	
Absolute	19	0.97	0.69	0.69	(	0.21	-	2.82	)	15	0.86	0.40	0.78	(	0.22	-	1.76	)	**0.681**			130
Eosinophils																						
Differential	19	7.89	5.35	8.0	(	1.0	-	21.0	)	15	12.47	7.01	12.0	(	4.0	-	28.0	)	0.039	32	-2.16	
Absolute	19	0.75	0.54	0.69	(	0.12	-	1.92	)	15	0.89	0.56	0.63	(	0.34	-	2.30	)	**0.376**			116.5
Basophils																						
Differential	17	0.41	0.71	0.0	(	0.0	-	2.0	)	15	0.13	0.35	0.0	(	0.0	-	1.0	)	**0.249**			150
Absolute	17	0.04	0.08	0.00	(	0.00	-	0.23	)	15	0.01	0.03	0.00	(	0.00	-	0.11	)	**0.21**			152
H: L ratio	19	0.26	0.76	0.48	(	0.24	-	4.13	)	14	0.43	1.90	0.83	(	0.39	-	6.75	)	0.006			59
CK	18	968.3	619.2	881	(	198	-	2484	)	14	1507.6	715.0	1580	(	411	-	3135	)	0.03	30	-2.28	
AST	18	197.2	62.0	183	(	114	-	351	)	14	277.9	129.1	225	(	183	-	594	)	0.012			59
ALT	18	7.3	2.8	7	(	3	-	13	)	14	8.6	4.3	7	(	5	-	20	)	**0.538**			109.5

ALP	18	73.3	19.6	70	(	44	-	116	)	13	87.5	37.3	80	(	35	-	174	)	**0.336**			92.5
Bilirubin T	18	2.04	0.53	2.0	(	1.0	-	2.9	)	14	2.25	0.47	2.1	(	1.6	-	3.0	)	**0.513**			108.5
BUN	18	18.60	3.67	18.8	(	12.0	-	25.4	)	14	20.04	3.92	19.6	(	14.9	-	31.0	)	**0.293**	30	-1.07	
Bile acids	18	2.1	1.2	2	(	0	-	5	)	14	1.5	0.5	2	(	1	-	2	)	**0.158**			161
Uric acid	18	0.070	0.018	0.072	(	0.033	-	0.106	)	14	0.086	0.017	0.084	(	0.051	-	0.115	)	0.017	30	-2.53	
Glucose FI-Ox	18	2.55	0.52	2.6	(	1.4	-	3.4	)	13	2.90	0.52	2.8	(	2.0	-	3.9	)	**0.074**	29	-1.85	
Cholesterol	18	6.77	3.28	7.0	(	2.6	-	15.0	)	14	3.75	0.91	3.6	(	2.3	-	5.4	)	0.006			199.5
Triglyceride	18	10.53	8.93	9.4	(	1.1	-	30.9	)	14	2.05	1.04	1.9	(	0.8	-	4.8	)	0.003			205
Na	18	149.9	3.3	150	(	144	-	156	)	14	153.9	3.8	153	(	149	-	160	)	0.004	30	-3.16	
K	18	3.87	0.47	4.0	(	2.4	-	4.5	)	14	3.73	0.35	3.7	(	2.9	-	4.3	)	**0.152**			164
Cl	18	118.6	5.8	119	(	108	-	126	)	14	122.9	4.3	123	(	117	-	130	)	0.02	29.97	-2.45	
Total Protein	18	46.7	8.5	46	(	32	-	68	)	14	42.4	4.4	44	(	34	-	49	)	**0.071**	26.7	1.88	
Albumin	18	14.3	2.5	15	(	10	-	20	)	14	12.4	1.7	13	(	8	-	14	)	0.019	30	2.49	
Globulin	18	32.4	6.07	31	(	22	-	48	)	14	30.0	3.2	31	(	26	-	35	)	**0.164**	26.98	1.43	
Ca	18	3.32	1.50	3.28	(	1.58	-	6.43	)	14	1.87	0.26	1.80	(	1.59	-	2.45	)	0.009			195.5
P	18	2.39	0.87	2.08	(	1.11	-	3.76	)	14	1.68	0.39	1.70	(	0.97	-	2.28	)	0.034			182
Ca: P	18	1.38	0.37	1.40	(	0.95	-	2.33	)	14	1.15	0.25	1.13	(	0.81	-	1.65	)	**0.111**			168.5
Mg	18	3.53	0.70	3.45	(	2.66	-	4.97	)	14	3.26	0.62	3.09	(	2.55	-	4.57	)	**0.305**			153.5
Fe	18	8.82	4.28	7.9	(	2.7	-	15.8	)	14	4.21	1.30	4.4	(	1.5	-	6.1	)	0.002			209.5
LDH	18	594.8	167.7	536	(	414	-	959	)	14	768.9	164.3	793	(	525	-	1009	)	0.005			51.5
GLDH	18	29.92	19.96	22.8	(	6.9	-	80.1	)	14	36.04	40.58	19.2	(	8.7	-	156.2	)	**0.779**			134
In-house PCV	21	39.3	2.7	39	(	35	-	45	)	15	37.9	2.5	38	(	34	-	43	)	**0.106**	34	1.66	
Total solids	20	59.7	17.8	56	(	34	-	102	)	15	45.3	6.1	48.0	(	31	-	54	)	0.017			222
Chem8+ Na	6	146.5	4.0	146	(| 143 | - | 154 |)													
Chem8+ K	6	4.02	0.29	4.1	(| 3.6 | - | 4.4 |)													
Chem8+ Cl	6	118.5	2.7	118	(| 117 | - | 124 |)													
Chem8+ Glucose	6	2.40	0.15	2.5	(| 2.1 | - | 2.5 |)													
Chem8+ BUN	6	19.65	2.73	19.1	(| 16.6 | - | 23.8 |)													
Chem8+ HCT	6	28.5	2.5	29	(| 25 | - | 31 |)													

[1] all WBC, differential and absolute values are estimated

[2] No I-stat comparisons available due to no male foraging I-stat results.

Appendix 15: Comparison of analytes for nesting flatback turtles at Thevenard Island and Eighty Mile Beach rookeries using parametric and non-parametric methods.

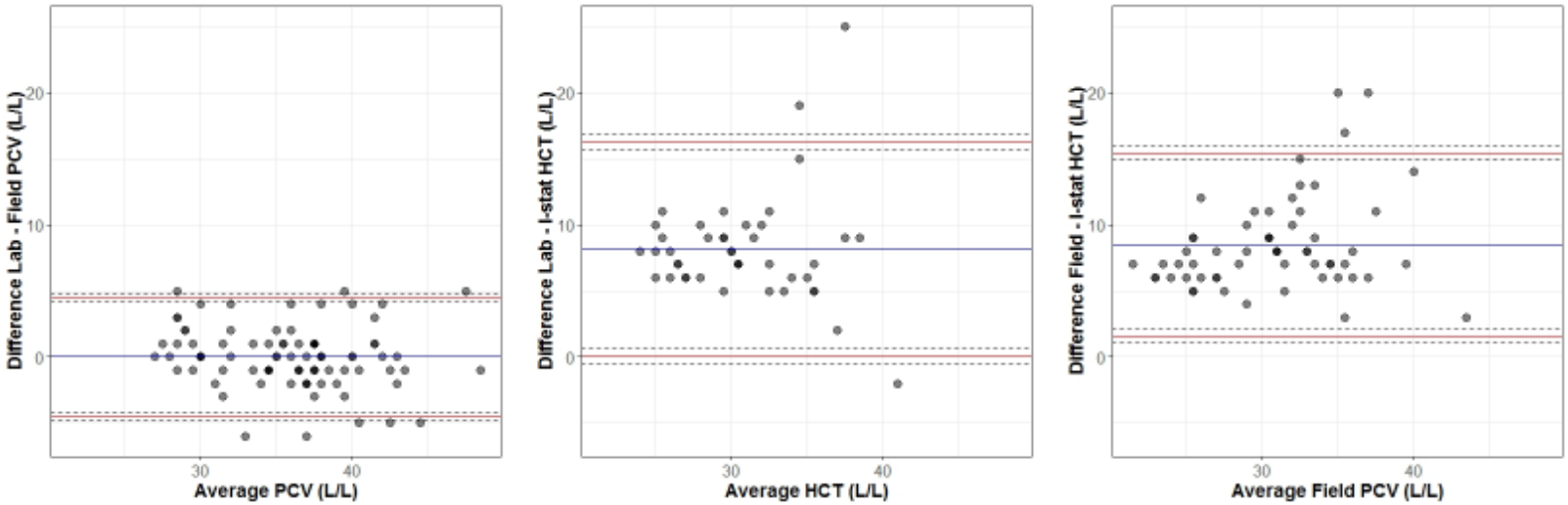
Analyte[1,2]	Eighty Mile Beach					Thevenard Island					p	df	t	W
	n	Mean +/- SD OR Median (10-90% percentiles)				n	Mean +/- SD OR Median (10-90% percentiles)							
PCV	30	34	(	28	- 42)	33	35	(	30	- 42)	**0.287**			417.5
Hb	30	103	(	95	- 124)	33	113	(	98	- 128)	0.038			343.5
RBC	29	0.20	(	0.19	- 0.32)	33	0.22	(	0.14	- 0.40)	**0.772**			458
MCHC	30	328	(	244	- 343)	33	318	(	300	- 337)	**0.591**			534.5
MCH	28	473	(	306	- 591)	31	450	(	320	- 635)	**0.897**			443
MCV	29	1500	(	952	- 2288)	32	1446	(	950	- 1995)	**0.696**			491.5
WBC	37	7.38 +/- 2.74				43	7.97 +/- 2.90				**0.356**	78	-0.93	
Heterophils														
Differential	40	51.13 +/- 13.05				44	58.98 +/- 18.92				0.029	76.65	-2.23	
Absolute	37	3.33	(	1.86	- 5.89)	43	4.59	(	2.26	- 7.27)	0.04			582.5
Lymphocytes														
Differential	40	29.0 +/- 11.82				44	24.75 +/- 16.54				**0.177**	77.82	1.36	
Absolute	37	2.12	(	0.87	- 3.37)	43	2.25	(	0.25	- 4.08)	**0.65**			843
Monocytes														
Differential	40	4.5	(	1.0	- 9.0)	44	4.0	(	1.0	- 10.7)	**0.829**			904.5
Absolute	37	0.34	(	0.06	- 0.67)	43	0.28	(	0.08	- 0.82)	**0.908**			783
Eosinophils														
Differential	40	14.8 +/- 5.69				44	11.32 +/- 6.55				0.011	82	2.59	
Absolute	37	1.05 +/- 0.57				43	0.87 +/- 0.58				**0.171**	78	1.38	
Basophils														
Differential	38	0.0	(	0.0	- 0.0)	44	0.00	(	0.0	- 0.0)	na			836
Absolute	35	0.00	(	0.00	- 0.00)	43	0.00	(	0.00	- 0.00)	na			752.5
H : L ratio	38	1.67	(	0.78	- 3.43)	33	1.68	(	0.62	- 6.02)	**0.931**			635
CK	42	367	(	228	- 1649)	42	592	(	220	- 1892)	**0.167**			727
AST	43	193.6 +/- 54.87				42	176 +/- 63.74				**0.15**	83	1.45	
ALT	44	6	(	4	- 11)	39	5	(	2	- 8)	<0.001			1227
ALP	40	54	(	37	- 74)	42	53	(	38	- 69)	**0.587**			899
Bilirubin T	44	2.0	(	1.0	- 3.5)	42	3.1	(	2.0	- 6.0)	<0.001			351
BUN	44	2.0	(	1.2	- 4.3)	42	1.3	(	0.7	- 2.0)	<0.001			1491
Bile acids	44	1	(	1	- 2)	40	1	(	1	- 2)	**0.171**			747
Uric acid	44	0.056	(	0.044	- 0.078)	42	0.068	(	0.046	- 0.083)	0.023			660.5
Glucose														
Li-Hep	28	4.8 +/- 0.68				20	4.23 +/- 0.66				0.005	46	2.92	
Fl-Ox	16	3.42 +/- 1.03				22	3.98 +/- 0.75				**0.075**	36	-1.84	
Cholesterol	44	7.1	(	4.4	- 9.2)	42	6.8	(	4.9	- 11.0)	**0.514**			848
Triglyceride	44	5.2	(	2.1	- 13.0)	42	8.7 +/- 2.86				0.004	75.07	-2.94	603
Na	44	154	(	149	- 158)	42	153	(	150	- 156)	**0.418**			1017.5
K	44	4.89 +/- 0.41				42	4.46 +/- 0.39				<0.001	84	4.95	
Cl	44	117	(	110	- 119)	42	108	(	104	- 112)	<0.001			1693.5
Total Protein	44	42.9 +/- 7.12				42	48.4 +/- 5.28				<0.001	84	-4.07	
Albumin	44	13	(	10	- 17)	42	14	(	13	- 18)	<0.001			536
Globulin	44	29.9 +/- 4.77				42	33.6 +/- 3.82				<0.001	84	-4.04	
Ca	44	3.64	(	2.70	- 5.61)	42	3.87	(	2.77	- 4.99)	**0.746**			886
P	44	3.70 +/- 0.72				42	3.3 +/- 0.83				0.018	84	2.42	

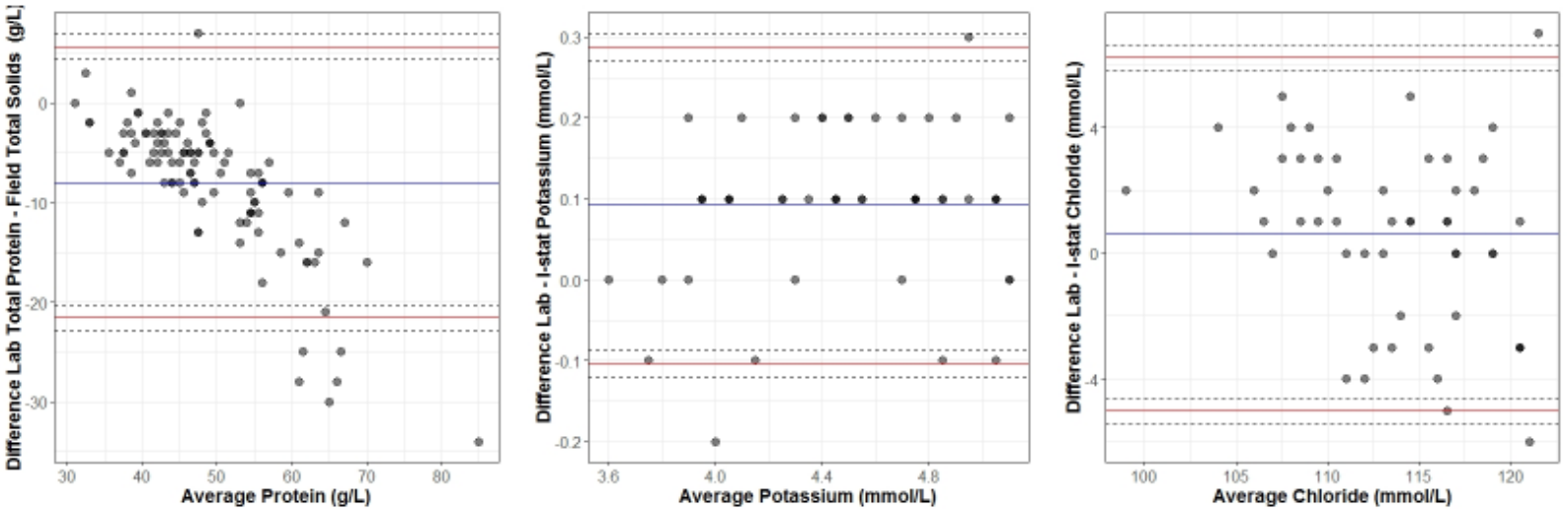
Ca: P	44	1.03	(	0.83	-	1.25	)	42	1.19	(	0.95	-	1.57	)	<0.001			529.5
Mg	43	4.39 +/- 0.65						42	4.06 +/- 0.65						0.022	83	2.34	
Fe	44	5.0	(	3.3	-	8.7	)	42	9.0	(	7.0	-	12.0	)	<0.001			237
LDH	44	477	(	332	-	932	)	42	551	(	394	-	937	)	**0.117**			742
GLDH	6	27	(	17	-	88	)	21	22	(	6	-	97	)	**0.28**			99
In-house PCV	56	35	(	28	-	46	)	38	36	(	28	-	41	)	**0.847**			1089.5
Total solids	64	48	(	40	-	64	)	57	56	(	47	-	73	)	<0.001			1032
Chem8+ Na	39	148	(	145	-	150	)	24	147	(	143	-	148	)	0.009			651.5
Chem8+ K	39	4.68 +/- 0.37						24	4.22 +/- 0.32						<0.001	61	5.09	
Chem8+ Cl	39	116.1 +/- 3.14						24	108.1 +/- 3.76						<0.001	61	9.03	
Chem8+ Glucose	39	3.9	(	2.4	-	4.8	)	24	3.8	(	2.6	-	4.5	)	**0.532**			512.5
Chem8+ BUN	39	0.5	(	0.5	-	4.0	)	24	0.5	(	0.5	-	0.5	)	<0.001			686
Chem8+ HCT	39	25.0 +/- 3.55						24	29.0 +/- 5.25						0.002	36.03	-3.38	

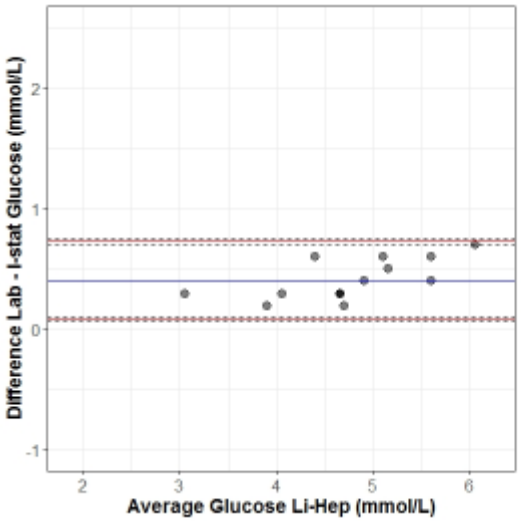
[1] all WBC, differential and absolute values are estimated

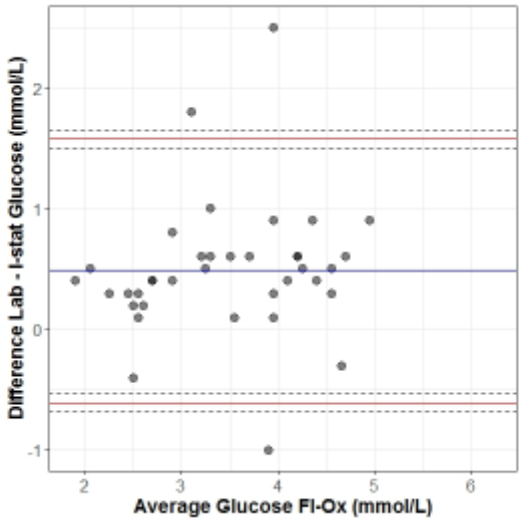
[2] No GLDH comparisons available due to no nesting GLDH values

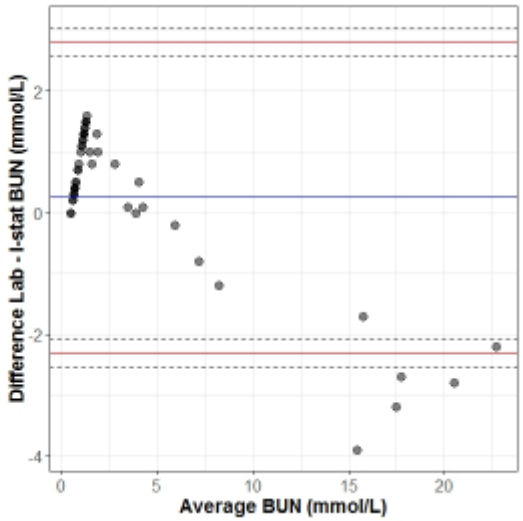
Appendix 16: Bland-Altman plots for combined flatback measurement methods with non-parametric differences including final PCV excluding >20% PCV values (n=88), HCT (n=43), Field PCV (n=60), Protein (n=110), Chloride (n=51), Potassium (n=51), Glucose Fl-Ox (n=37) (and Glucose Li-Hep n=14 to facilitate comparison) and BUN (n=51) showing the mean or bias (blue line) and limits of agreement (red line) with confidence intervals (dotted line). These graphics show the results from different measurement methods for PCV (Laboratory and field), BUN (laboratory and i-STAT) and Chloride (laboratory and i-STAT) were not significantly different and the methods can be used interchangeably.




Difference Lab Total Protein - Field Total Solids (g/L)
0
-10
-20
-30
30
40
50
60
70
80
Average Protein (g/L)
Difference Lab - I-stat Potassium (mmol/L)
0.3
0.2
0.1
0.0
-0.1
-0.2
3.6
4.0
4.4
4.8
Average Potassium (mmol/L)
Difference Lab - I-stat Chloride (mmol/L)
4
0
-4
100
105
110
115
120
Average Chloride (mmol/L)


Difference Lab - I-stat Glucose (mmol/L)
2
1
0
-1
2
3
4
5
6
Average Glucose Li-Hep (mmol/L)


Difference Lab - I-stat Glucose (mmol/L)
2
1
0
-1
2
3
4
5
6
Average Glucose FI-Ox (mmol/L)


Difference Lab - I-stat BUN (mmol/L)
2
0
-2
-4
0
5
10
15
20
Average BUN (mmol/L)

Appendix 17: Sea turtle species age-classes and approximate sizes based on sea turtle populations from Australia and overseas (Wyneken et al. 2006, Limpus 2007, 2008a, b, c, 2009, Norton and Wyneken 2015).

Species	CCL (cm)					Weight (kg)	Age (yrs)
	Hatchling	Post-hatchling	Juvenile	Sub-adult	Adult	Adult female	Adult female
Green	<5	<20	40-65*	65-90*	>90 (av. 100)*	96-186	28-40
Hawksbill	<5	<30	~35*	~60	>75 (av. 80)*	78-91	-
Loggerhead	<5	<45*	~80*	<90	> 90 (av. 100)*	170-182	29-30
Flatback	<7	≥7*		≤85*	> 85 (av. 90)*	-	-
Olive ridley	<5		>50	<65*	<80 (av. 70)	35-45	13

* Indicates species and age-class observed in this study.

Appendix 18: 'Cause of stranding' and 'Cause of mortality' data for morbidity and mortality cases (n=75).

ID	Cause of Stranding	Cause of Mortality
1	Trauma	Trauma
2	Mass mortality event	Unknown
3	Mass mortality event	Unknown
4	Mass mortality event	Unknown
5	Chronic debilitation	Spirorchiidiasis
7	Trauma	Trauma
8	Chronic debilitation	Osmoregulatory disorder
9	Chronic debilitation	Spirorchiidiasis
10	Trauma	Trauma
11	Trauma	Trauma
12	Buoyancy disorder	GI impaction
13	Buoyancy disorder	Unknown
14	Unknown	Spirorchiidiasis
15	Trauma	Unknown
16	Mass mortality event	Unknown
17	Unknown	Microbiological infectious disease
18	Trauma	Spirorchiidiasis
19	Mass mortality event	Unknown
20	Mass mortality event	Unknown
21	Mass mortality event	Microbiological infectious disease
22	Mass mortality event	Microbiological infectious disease
23	Mass mortality event	Unknown
24	Mass mortality event	Unknown
25	Mass mortality event	Microbiological infectious disease
26	Chronic debilitation	Spirorchiidiasis
27	Unknown	Spirorchiidiasis
28	Unknown	Spirorchiidiasis
29	Chronic debilitation	GI foreign body
30	Trauma	Trauma
31	Chronic debilitation	Osmoregulatory disorder
32	Fibropapillomatosis	Spirorchiidiasis
33	Entanglement	Fibropapillomatosis
34	Trauma	Unknown
35	Trauma	Microbiological infectious disease
36	Cold stunning	Endoparasitosis
37	Trauma	Unknown
38	Cold stunning	Endoparasitosis
39	Unknown	Metabolic disorder

40	Entanglement	Trauma
41	Unknown	Unknown
42	Chronic debilitation	Spirorchiidiasis
43	Chronic debilitation	Spirorchiidiasis
44	Chronic debilitation	Spirorchiidiasis
45	Trauma	Trauma
46	Buoyancy disorder	Spirorchiidiasis
47	Chronic debilitation	Spirorchiidiasis
48	Trauma	Trauma
49	Unknown	Systemic inflammation
50	Trauma	Spirorchiidiasis
51	Cold stunning	Pneumonia
52	Chronic debilitation	Spirorchiidiasis
53	Trauma	Trauma
54	Chronic debilitation	Spirorchiidiasis
55	Chronic debilitation	Spirorchiidiasis
56	Unknown	Spirorchiidiasis
57	Chronic debilitation	Spirorchiidiasis
58	Chronic debilitation	Spirorchiidiasis
59	Chronic debilitation	Spirorchiidiasis
60	Cold stunning	Endoparasitosis
61	Cold stunning	Endoparasitosis
62	Cold stunning	Endoparasitosis
63	Cold stunning	Pneumonia
64	Cold stunning	Pneumonia
65	Cold stunning	Pneumonia
66	Cold stunning	Systemic inflammation
67	Cold stunning	Endoparasitosis
68	Cold stunning	Pneumonia
69	Cold stunning	Systemic inflammation
70	Cold stunning	Endoparasitosis
71	Chronic debilitation	GI impaction
72	Chronic debilitation	Spirorchiidiasis
73	Chronic debilitation	Spirorchiidiasis
74	Chronic debilitation	Endoparasitosis
75	Chronic debilitation	Spirorchiidiasis
76	Trauma	Trauma

Appendix 19: Flatback turtle and sea snake bacterial culture and *S. iniae* PCR results.

ID	Species	Specimen	*S. iniae* culture	*S. iniae* PCR
AS-17-2548	Flatback turtle WT2 (*Natator depressus*)	Liver	Negative	Indeterminate*
AS-17-2548 AS-20-1550	Flatback turtle WT22 (*N. depressus*)	Liver Spleen Kidney Heart	Negative **2 colonies** **Heavy growth** **Moderate growth**	Negative Negative Negative **Positive**
AS-17-2548 AS-20-1550	Black-ringed sea snake WS1 (*Hydrelaps darwinensis*)	Liver Spleen Kidney Heart	Negative Negative Negative Negative	Negative Negative Indeterminate* Negative
AS-17-5645	Flatback turtle WT19 (*N. depressus*)	Frozen organs	Negative	Negative
AS-17-5645	Flatback turtle WT20 (*N. depressus*)	Liver	Negative	**Positive**
AS-17-5645	Flatback turtle WT23 (*N. depressus*)	Liver/heart	Negative	Negative
AS-17-5645	Olive sea snake WS2 (*Aipysurus laevis*)	Liver Kidney	Negative Negative	Negative Negative
AS-17-5645 AS-20-1550	Flatback turtle WT21 (*N. depressus*)	Liver Kidney Heart	**Moderate growth** **Moderate growth** Negative	**Positive** Negative **Positive**
AS-17-5645 AS-20-1550	Flatback turtle WT24 (*N. depressus*)	Liver Kidney Heart	Negative Negative Negative	Negative Negative Negative
AS-17-5645 AS-20-1550	Flatback turtle WT25 (*N. depressus*)	Liver Kidney Heart	Negative **Moderate growth** Negative	**Positive** Negative **Positive**
AS-17-5645 AS-20-1550	Black-ringed sea snake WS4 (*H. darwinensis*)	Liver Kidney Heart	Negative Negative Negative	Negative Negative Negative
AS-17-5645 AS-20-1550	Olive sea snake WS6 (*A. laevis*)	Liver Kidney	Negative Negative	**Positive** **Positive**
AS-20-1550	Flatback turtle WT4 (*N. depressus*)	Liver Brain	Negative Negative	Negative Negative
AS-20-1550	Olive sea snake WS3 (*A. laevis*)	Liver Heart	Negative Negative	Negative Negative

* PCR yielded a weakly staining band resulting in an inconclusive result.