The Impact of Health and Environmental factors on a Population of Mekong River Irrawaddy Dolphins (Orcaella brevirostris) in Cambodia

By

Verné Dove

BVSc (Hons), BAnimSc, MVS (Cons. Med.), MVS (Vet. Surveil.), Dip Cons.

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DECLARATION

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary institution.

Dr Verné Dove.

BVSc (Hons), BAnimSc, MVS (Cons. Med.), MVS (Vet. Surveil.), Dip Cons.

ABSTRACT

Background

The critically endangered Mekong River Irrawaddy dolphin (MRID) (*Orcaella brevirostris*) population, estimated to comprise 85 individuals in 2010, is at risk of extirpation due to a high level of mortality, particularly in calves, and a very low recruitment level (<1%) into the population; however existing studies have failed to identify the causes of mortality in this population. In this study, a retrospective study was conducted to better understand the causes of mortality in different age classes, so that potentially mortality levels can be mitigated and recruitment rates improved.

Methods

Mortality data from 2001-2010 were analysed to identify mortality trends in 102 MRIDs. Samples were also collected from 41 necropsied dolphins and subjected to microbiological, histopathological, genetic and toxicological analyses to identify and quantify the threats to this population. Additionally, a study of skin lesions using photo-identification data of live MRIDs from 2007 to 2010 was conducted.

Results

In this study, significantly high levels of immuno-toxic pollutants, particularly DDT and mercury; genetic factors, specifically genetic drift and outbreeding depression; and disease and immunosuppression; were all contributing towards the population's decline.

Dead calves were 15 times (95%CI 2.4, 88.1, p=0.001) more likely to have a localised gangrenous, blue/black neck lesion than dead adult dolphins. These lesions were clinically,

pathologically and microbiologically similar to necrotising fasciitis of humans. In contrast, adults were 17 times (95%CI of 1.8, 166.1, p=0.006) more likely to have evidence of interaction with fishery activities than juveniles and calves.

The incidence of mortality in adults over a three year period when gillnets were used was 18.3% (95%CI 11.6, 26.9), and this reduced to 6.7% (95%CI 2.5, 14.1) when restrictions on the use of gillnets were introduced. The risk of dying during the three years with gillnets was 2.7 times (95%CI 1.1, 6.5) higher than the period when gillnet use was restricted (p=0.016). In contrast, there was no significant difference in mortality for calves for years with and without gillnet restrictions (p=0.39).

Skin lesions were common in live MRIDs being detected in 35 of 84 individuals observed (41.6%; 95%CI 31.1, 52.2) over a three year period. However, the mean prevalence of MRIDs photographed with skin lesions during 11 surveys was 7% (95%CI 1.9, 16.7), with a significant difference found in the prevalence of skin lesions in MRIDs from pools 1 and 9 (Fisher's exact test p=0.002).

In necropsied MRIDs (n=8), significantly more calves 100% (95%CI 47.8, 100) had neck lesions and associated neck swelling with wet gangrenous musculature than adults (0%; 95%CI 0.0, 70.8) (p=0.018). The three most frequent bacteria cultured in 13 dolphins examined with neck lesions were *Aeromonas hydrophila* (54%, 95%CI 25.1, 80.8), *Plesiomonas shigelloides* (46%, 95%CI 19.2, 74.9), and ß-haemolytic Group C *Streptococcus* spp. (23%, 95%CI 5, 53.8). Toxicological analysis in a larger sample set (n=20), revealed

that the levels of mercury (Hg) and methylmercury (MeHg) were significantly higher in the adult dolphins sampled than in calves (11.21µg/g vs. 1.51µg/g, p=0.019; 7.23µg/g vs. 0.72, p=0.033, respectively). In contrast, calves had significantly higher levels of liver copper (Cu) than adults (40.72µg/g vs. 3.49µg/g, p=0.0005). Adults also had a significantly higher level of Hg (11.21 µg/g vs. 0.91 µg/g, p=0.026) and MeHg (7.23 µg/g vs. 0.17 µg/g, p=0.006) than juveniles. The mean molar selenium:mercury ratio was significantly higher in calves (2.27, p=0.039) and juveniles (2.94, p=0.007) than in adults (0.95). The level of zinc (Zn) in the liver of adults with neck lesions (28.7µg/g) was significantly lower (p=0.049) than that of adults without evidence of neck lesions (65.17µg/g). The concentrations of organochlorines in the blubber of MRID decreased in the order of DDTs > PCBs > CHLs > HCB > PBDEs > Dioxins > OC. The level of ∑Dioxins was significantly higher in adults (2.27TEQ) than in calves (1.15TEQ, p=0.004), and also significantly higher in juveniles (3.57TEQ) than in calves (p=0.0094). The levels of PCBs in adults (346.9ng/g) and in juveniles (705.4ng/g) were significantly higher than in calves (142.75ng/g) (p=0.019, p=0.012, respectively). Adult females had higher ∑Dioxin levels (61.3pg/g; 2.27TEQ) than female (3.7pg/g, p=0.0017; 1.15 TEQ p=0.02) and male calves (2.8pg/g, p=0.003; 0.99 TEQ, p=0.01). Juvenile females had significantly higher ∑Dioxins (3.57TEQ) and ∑PCBs (705.4ng/g) than female (1.15TEQ, p=0.02; 163.2ng/g, p=0.016) and male calves (0.99TEQ, p=0.01; 142.8ng/g, p=0.03) respectively. Adult males had significantly higher PCB levels (606.8ng/g) than female (163.2ng/g, p=0.01) and male calves (142.8 ng/g, p=0.03). The levels of ∑PBDE, ∑CHLDs, ∑DDT and ∑HCB were all significantly higher in MRIDs than in the Chilika Lake Irrawaddy dolphins from the study of Kannan et al. (2005) (p=0.0007).

Phylogenetically the MRIDs appear to be an evolutionary significant unit (ESU), with six new haplotypes identified in this study. The average nucleotide diversity (π) was 0.001 (± SD

0.0001) and the average haplotype diversity (*h*) was 0.812 (± SD 0.333). 34% (n=11) of the necropsied MRIDs had Infrequent/Rare (I/R) alleles, with an excess found in the adults compared to the calves, indicating a strong likelihood of genetic drift occurring. Paternity tests suggested reproductive failure, as only a few dolphins were breeding in the population and breeding females were genetically related.

Conclusions

It is concluded that two principal factors are driving the population decline. Firstly, the interaction of adults with commercial fishery activities and secondly, necrotising fasciitis associated with neck lesions in the calves. Furthermore, reproductive failure is a major contributing factor limiting recruitment into the population and ultimately affecting the population stability. This study highlights the need for urgent attention to save this population from certain extirpation.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

DEDICATION

I would like to dedicate this thesis to my amazing family, my husband Troy, and my three beautiful children, all of which were born during this PhD study; Jaidal, Trinity and Justis.

QUOTE

Two roads diverged in a wood, and I—I took the one less travelled by, and that has made all the difference. –Robert Frost, 1915.

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CHAPTER ONE

Introduction

1.1 Background

River dolphins and porpoises are among the most threatened mammals of the world (Ávila et al., 2018). This was highlighted by the recent extinction of the Baiji/Yangtze River dolphin (*Lipotes vexillifer*) in 2006 (Turvey et al., 2007; Veron et al., 2008; WWF, 2010), the first reported extinction of a dolphin species. This global threat arises from habitat loss, pollution, overfishing, incidental mortality from fisheries, boat collisions and disturbance or displacement by intensive vessel traffic (Veron et al., 2008). Freshwater populations of Irrawaddy dolphins (*Orcaella brevirostris*) are among the cetaceans at greatest risk of population extirpation and perhaps extinction (Jefferson and Smith, 2002; Kannan et al., 2005; Kreb and Budiono, 2005; Stacey and Leatherwood, 1997), with the Mekong River Irrawaddy dolphin (MRID) population considered to be the most threatened in this species (Beasley, 2007; Beasley et al., 2013; Ryan et al., 2011; Caballero et al., 2018a).

1.2 Irrawaddy dolphins (*Orcaella brevirostris*)

The Irrawaddy dolphin inhabits a wide range of coastal and riverine habitats from the far north-western Bay of Bengal to the east coast of Australia (Beasley et al., 2005; Jayasankar et al., 2011; Stacey and Leatherwood, 1997). These dolphins reside in some of the largest rivers and marine appended lakes in Southeast Asia, and coastal waters of the Indo-Pacific (Caballero et al., 2018a; Smith et al., 2003). Jefferson and Smith (2002) described this species as a facultative freshwater cetacean, occupying both fresh and near-shore marine

waters. There are five freshwater populations of *O. brevirostris*, three occurring in major Asian river systems (Beasley et al., 2009): the Ayeyarwady River in Myanmar (1,500 km upstream); the Mekong River in Cambodia and southern Laos People's Democratic Republic (Laos PDR) (690km upstream); and the Mahakam River/Semayang Lake system in Indonesia (560km upstream) (Stacey, 1996; Stacey and Leatherwood, 1997); and two populations in lakes: Songkla Lake, Thailand; and Chilika Lake, India (Beasley et al., 2009).

Globally, *Orcaella* are distributed in six countries of Asia and in Australia, although there are likely to be less than 7000 individuals belonging to the genus (Jayasankar et al., 2011). Asian (*O. brevirostris*) and Australian (*O. heinsohni*) members of the genus were designated as separate species in 2005 (Beasley et al., 2005). Beasley et al. (2005) concluded that such designation and differentiation, meant higher conservation priorities were required for the Asian populations of Irrawaddy dolphins, that now comprised far fewer individuals than previously thought (Caballero et al., 2018a). One of the major threats to *O. brevirostris* has been related to deterioration of their habitat due to anthropogenic events, in particular associated with intensive human use (Kannan et al., 2005; Reeves et al., 2003). All three riverine populations of *O. brevirostris* are in rapid decline, and all are facing potential extirpation in the near future (Beasley et al., 2005; Beasley, 2007).

1.2.1 Systematics and taxonomy

Orcaella brevirostris (Owen in Gray, 1866) belongs to the order Cetacea and the family Delphinidae. Its common name is the Irrawaddy dolphin in English or Ph'sout in Khmer and Pha'ka in Laotian.

Prior to the commencement of this study, little was known about the genetics of *O. brevirostris*, with only two peer-reviewed journal articles published (Beasley et al., 2005, Jayasankar et al., 2011). The genetic data analysed by Beasley et al. (2005) provided support for two clades within the Asian population: the Mekong River clade represented by dolphins from Cambodia and southern Laos PDR; and a clade containing dolphins from all other marine and freshwater sites from Thailand, Indonesia and the Philippines. In total, six haplotypes have been identified in the Asian *O. brevirostris* (Beasley et al., 2005). Although *O. brevirostris* is the recognised species in Asia, Beasley et al. (2005) and Jayasankar et al. (2011) postulated that there were genetic subdivisions within this population, and highlighted the need for further research to clarify if distinct genetic populations of *Orcaella* exist in different habitats, or if they were genetically similar. Beasley et al. (2005), however, found no morphological evidence to support the separation of *O. brevirostris* specimens from freshwater sites and those from marine habitats. Recently a genetics study by Krützen et al. (2018), using samples collected from the study reported in this thesis, provided evidence to support a monophyletic clade. In contrast, Caballero et al. (2018a) found that the MRIDs shared a haplotype with coastal dolphins from Thailand, refuting the findings of Krützen et al. (2018).

1.2.2 Status of *Orcaella brevirostris* in Cambodia and Laos PDR

The International Union for Conservation of Nature (IUCN) classified Cambodia's only freshwater dolphin, the MRID, as 'Critically Endangered' in 2004 (Minton et al., 2017; Smith and Beasley, 2004; Smith and Reeves, 2004). The MRIDs are also listed in the Appendix I of the Convention in International Trade of Endangered Species (CITES), and are protected in both Cambodia and Laos PDR (Ryan et al., 2011; Vibol et al., 2009).

Since the early 1990s the MRID population has experienced a high level of mortality, particularly in calves (Baird and Mounsouphom, 1994; 1997; Baird et al., 1994; Beasley, 2007; Beasley and Gilbert, 2005; Beasley et al., 2013; Dove et al., 2007; Stacey and Leatherwood, 1997). From 1990 until June 1996, a total of 31 dead dolphins were reported in the northern most dolphin pool (pool 9) in the Mekong River at the Laos PDR/Cambodia border (Baird and Mounsouphom, 1994, 1997; Baird et al., 1994). Subsequently, between 2001 and 2010, a further 106 dead dolphins were reported along a 200km stretch of the Mekong River in Cambodia, from Kampi in the province of Kratié to the Laos PDR border (Beasley, 2007; Beasley and Gilbert, 2005; Beasley et al., 2009; Dove, 2008; 2009; McLellan, 2010 pers. comm.; Reeves et al., 2009; Schnitzler et al., 2021), of which 68% were calves (Dove, 2009), and subsequently mortalities have continued to be reported (Limsong et al., 2017). Given the critically endangered status of this population, this mortality level is considered unsustainable (Beasley, 2007; Caballero et al., 2018a; Dove et al., 2007; Krützen et al., 2018; Ryan et al., 2011, Vibol et al., 2009). In addition, the probability of recruitment into the population (defined as calves reaching sexual maturity, at approximately 12 years of age, and then reproducing successfully) was estimated by Ryan et al. (2011) as extremely low (0.001, 95%CI 0, 0.017), based on annual data over a three year period. Due to the high mortality and low recruitment levels, along with an aging population, the MRID has been considered as the most threatened population of the freshwater Irrawaddy dolphins (Ryan et al., 2011), and at the current rate of decline, extirpation would appear inevitable.

1.2.2.1 Abundance

In Laos PDR and Cambodia, villagers have indicated that dolphin populations have been declining significantly since at least the 1970s, and in many places where dolphins used to be abundant year round they are now rarely seen (Baird and Mounsouphom, 1994). This declining trend was highlighted by Beasley (2007), who reported that the population was declining by 4.8% per year.

Unfortunately historical abundance data for the MRIDs does not exist; however, His Excellency (H.E.) Nao Thuok (Director of the Ministry of Fisheries in Cambodia) stated that before 1975 there were more than 1,000 dolphins. However, during the four-year Khmer Rouge regime at least five dolphins were killed per day in the Tonle Sap Great Lake (Nou Thouk, pers. comm.). Krützen et al. (2018) estimated that the current population size was only 5.2% of the ancestral population (≈1,630 dolphins). Similar historical population figures prior to the war have been reported by Cambodian villagers (Beasley, 2007); however no accurate historical population size is available. The first abundance estimates were reported by Baird and Beasley (2005) who considered that the total Irrawaddy dolphin population in the Mekong River basin in 1997 was less than 200. Subsequently, Beasley (2007) estimated that the population size was 127 (95%CI 108-146) in 2005; although on review of photo-identification studies from 2001-2005 and 2007 this estimate was reduced to 93 (95%CI 86-101) (Beasley et al., 2013). However, the last robust population abundance analysis carried out for this population using photo-identification studies from 2007 to 2010 estimated there were only 85 (95%CI 78-91) dolphins remaining in the Mekong River (Ryan et al., 2011), demonstrating a 58% reduction over the 13 years, and a 9% reduction from 2007 to 2010. Subsequent reports by the WWF arising from further photo-identification surveys, suggested that this declining trend was continuing (Channah et al., 2015). Overall, these abundance

studies show a clear declining trend of approximately 4.4% annually, which is consistent with that reported by Beasley (2007). Despite having no absolute historical abundance estimate, it is clear from the research conducted over the past two decades that this population is declining rapidly.

1.2.2.2 Distribution

The distribution of the MRID population has been greatly reduced from its historical range (Baird and Mounsouphom, 1994), with estimates of a tenfold reduction since the latter half of the 20th century (Smith and Jefferson, 2002; Beasley, 2007). The range is now primarily restricted to nine deep pool areas (Figure 1-1) in a 190 linear km stretch of the Mekong River from Kratie to the Khone Falls, just upstream of the Laos PDR-Cambodian border (Beasley et al., 2005). This, according to Beasley (2007), represents a contraction of 90% of the range in the dry season and 99% in the wet season. Historically, the range included the Sekong River 200km north of the Laos PDR-Cambodian border (Baird and Mounsouphom, 1997), the Tonle Sap Great Lake in Cambodia, and far downstream in the Mekong River into Vietnam (Baird and Mounsouphom, 1997; Jefferson and Smith, 2002; Lloze, 1973). No dolphins have been reported in the Tonle Sap Great Lake since 1997 (Baird and Beasley, 2005). Similarly, the freshwater MRIDs are now extirpated from the Mekong River in Vietnam (Ryan et al., 2011; Smith et al., 2007; Vibol et al., 2009) with Smith et al. (1997) unable to find any dolphins during their surveys conducted in 1996.

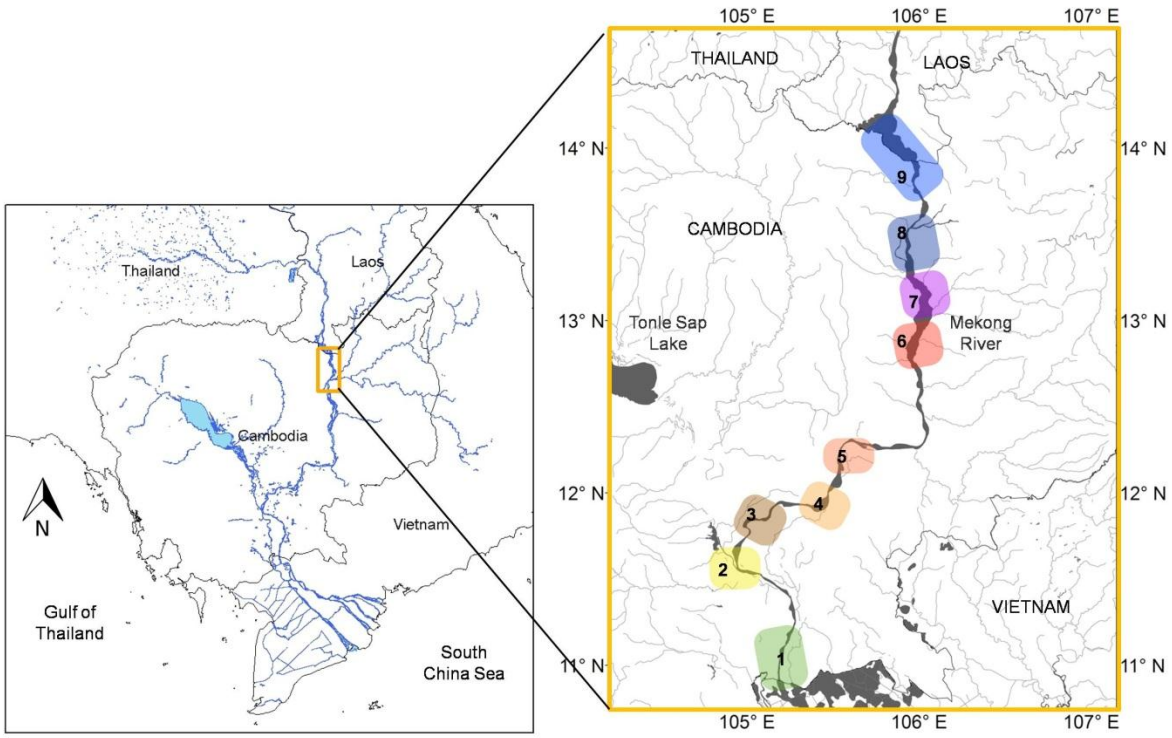


Figure 1-1: Mekong River Irrawaddy dolphin distribution in Cambodia/ Laos PDR. The map on the right displays the nine deep pool areas in the Mekong River.

1.2.2.3 Population structure

The MRID population is very age skewed, with the majority of the 85 individuals identified in 2010 by Ryan et al. (2011) using photo-identification mark-recapture methodology being adults, with many likely to be aged animals. Ryan et al. (2011) also found that few new animals were surviving to sexual maturity and thus being recruited into the population, with a very low probability of recruitment of only 0.001 (<1%), as mentioned earlier. It is believed that Irrawaddy dolphins can live for up to 30 years (Marsh et al., 1989), thus if very few calves have been recruited into the population since the late 1970s, the remaining dolphins are likely to be of advanced age, supporting the findings of Ryan et al. (2011). Photo-identification studies conducted from 2007-2010 (Dove et al., 2008; Ryan et al., 2011) indicated that the population appeared to be clustered into 4 groups: group A in pools 1, 2 and 3 (Figure 1-1); group B in pools 4 and 5; Group C in pools 6, 7, and 8; and group D in pool 9.

Similar groupings were reported by Krützen et al. (2018). Although in the wet season the Mekong River rises several metres, potentially allowing dolphins to travel between pools, photographic studies have failed to show evidence of mixing of any of the groups (Ryan et al., 2011). Despite this, Krützen et al. (2018) found no evidence of genetic structure in the MRIDs, suggesting population panmixia for the MRIDs.

1.2.2.4 Human impacts

Anthropogenic threats have emerged as a major factor responsible for deaths in the MRIDs. In the 1970s, dolphins were slaughtered in large numbers for oil (Jefferson and Smith, 2002). Between 1975 and 1979, the Khmer Rouge, Cambodian-Vietnamese and Indochina wars were believed to have taken a heavy toll on the dolphin population in the Sekong River sub-basin and Mekong River basin, as the MRIDs were used as target practice by the Vietnamese soldiers and were likely killed from aerial bombing by the USA air force (Baird and Mounsouphom, 1994; 1997). In the 1980s and 1990s, intensive fishing practices using gillnets and explosives emerged as another major threat to the population, and were responsible for a large number of dolphin mortalities (Baird and Mounsouphom, 1994; 1997; Baird et al., 1994; Beasley, 2007). In 1994, eight MRIDs were captured and sent to Safari World in Thailand (Perrin et al., 1996), with a subsequent report of at least another eight being captured in 2002 for display at Koh Kong International Resort, Thailand (Smith and Reeves, 2004). Since being upgraded to a CITES appendix I species in 2004, this direct human impact on the population has greatly reduced.

Mortality investigations since the 1990s have revealed that gillnet entanglement are responsible for a proportion of the dead dolphins (Baird and Mounsouphom, 1994; Beasley

and Gilbert, 2005; Beasley, 2007). In contrast, although Baird and Mounsouphom (1994) recovered and reported on several dead MRID carcasses in 1992 and 1993, only a small proportion had evidence of gillnet entanglement. These authors and Beasley (2007) concluded that the cause for the other mortalities was mostly unknown. Although Baird and Mounsouphom (1994) noted that gillnets were a potential threat to the MRIDs, most of the time MRID appeared to be able to detect and avoid these nets.

The MRIDs are also threatened by the proposal to develop a dam for hydroelectric power generation in pool 2, where a large proportion of the MRIDs are found. Construction of such a dam would certainly hasten the extirpation of the MRID population (Baird and Mounsouphom, 1994; Brownell et al., 2019; Dove 2009; WWF et al., 2014; Vibol et al., 2009) through modifying or eliminating the habitat of the MRID (Limsong et al., 2017). Since the construction of the Don Sahong dam began in 2014, the dolphins in pool 9 on the Laos PDR/Cambodia border have decreased from seven (Dove et al., 2008; Ryan et al., 2011) to only three individuals (Anonymous, 2016; Thomas and Gulland, 2017), highlighting the potential impact that dams can have on the survivability of the MRIDs.

1.2.2.5 Conservation initiatives

Conservation efforts for the MRIDs began in 2001, with Beasley (2007) establishing the Mekong Dolphin Conservation Program (MDCP) in collaboration with researchers from the Wildlife Conservation Society (WCS) and the Ministry of Fisheries Administration (FiA) (subsequently renamed the Ministry of Agriculture Forestry and Fisheries - MAFF). From 2001 to 2005, the MDCP primarily focused on research on the MRID's demographics and distribution in Cambodia. This work resulted in the development of the Cambodian Mekong

Dolphin Conservation Strategy published by MAFF in 2005 (Kreb et al., 2010; Smith et al., 2007). In 2006, the World Wildlife Fund for Nature (WWF-Cambodia) established the Cambodian Mekong Dolphin Conservation Project (CMDCP) to implement this strategy. I became the principal veterinary researcher from 2006-2011, with collaboration between WWF-Cambodia, WCS-Cambodia, Murdoch University, Volunteers for International Development (ViDA) and MAFF. The Cambodian Rural Development Team (CRDT), which promotes alternative livelihood development along the Mekong River, became a partner organisation of the CMDCP in 2006 (Vibol et al., 2009; Kreb et al., 2010). The CMDCP focused primarily on mortality investigation, in addition to continuing photo-identification studies to build knowledge about the population structure, movement, distribution, abundance, and behaviour of the MRID (Dove et al., 2007; Dove et al., 2008), and also undertook programs to increase conservation awareness in schools, villages and monasteries.

The Commission for Conservation and Development of Mekong River Dolphin Eco-tourism Zone (DC) was initiated in 2006 by the Prime Minister (H.E. Hun Sen), with an objective to develop ecotourism in Cambodia and to mitigate dolphin mortality. The DC board included relevant government stakeholder representatives (e.g. Tourism, MAFF, Military police, and Provincial Governors) (Vibol et al., 2009). The DC is a powerful body that supervises all Ministries and reviews, approves all proposed legislation from Ministries prior to submission to parliament, and sits within the Council of Ministers (Vibol et al., 2009; Kreb et al., 2010). The DC was given extensive power that enabled it to override all authorities and previous laws relating to dolphin conservation; and to direct Ministries to follow procedures and activities set down by the DC (Vibol et al., 2009). This allowed the DC to established 16 ranger stations along the Mekong River and employ 64 official river guards to protect the MRIDs (Vibol et al., 2009). The DC was also instrumental in getting the Royal Government

of Cambodia to impose a ban on the use of gillnets in the Mekong River from Kampi (pool 1) to the Cambodian-Laos PDR border (pool 9). However, the gillnet ban was not incorporated into fishery legislation, that permitted the use of gillnets with a mesh size of greater than 1.5cm and less than 15cm, despite all cetaceans in Cambodia being protected under fisheries legislation and a royal decree. Consequently, the ban could not be adequately enforced; however it was believed to have reduced fisheries related dolphin mortality (Vibol et al., 2009).

Corruption in developing nations may result in underestimation of the true incidence of mortality. For example, following the banning of gillnets in 2008, dead MRIDs, particularly calves, were often buried by the river guards due to fear of government repercussions, with several carcasses confirmed from burial sites by the CMDCP (Vibol et al., 2009). In addition, abundance figures and calf survival were often inflated in official government reports (Lovgren, 2019) to demonstrate that the conservation measures implemented by the government were working. Such practices, make the assessment of data and conservation success in a developing country challenging.

1.2.2.5.1 Scientific conflict

In 2009, an international workshop was hosted by WWF-Cambodia and MAFF in Cambodia. At this meeting and subsequently, there was extensive scientific debate on the aetiology of mortality and population decline in the MRIDs (CSG, 2012a; b; Dove, 2008; Gulland, 2009; 2014; CSG, 2012; Krützen et al., 2018; McLellan, 2010 pers.comm.; Reeves et al., 2009; Schnitzler et al., 2021; Smith, 2017; Thomas and Gulland, 2017; Wells, 2014; WWF et al., 2012; 2014). Following this workshop, a Conservation Task Force, consisting of 46 experts

and international scientists representing the IUCN Cetacean Specialist Group (CSG) and Veterinary Specialist Group (VSG), was established to review the documented mortality data for the period from 2001-2006 of Beasley and Gilbert (2005) and Beasley (2007), and from 2006-2010 of Dove (2008; 2009) and Ryan et al. (2011). Since 2009, this task force has worked with WWF-Cambodia and the Cambodian Government to coordinate and develop MRID conservation efforts (Krützen et al., 2018) and has also conducted three additional workshops resulting in the production of a number of summary reports (CSG, 2012a; 2012b; Gulland, 2009; 2014; Limsong et al., 2017; Reeves et al., 2009; Smith, 2017; Thomas and Gulland, 2017; Wells, 2014; WWF et al., 2012; WWF et al., 2014), external review reports (McLellan, 2010 pers. comm.; Siebert and Das, 2010), and two peer reviewed publications (Krützen et al., 2018; Schnitzler et al., 2021). However, for almost two decades there has been extensive debate and divergent opinions on the role of disease, immunosuppression, environmental contaminants, genetic factors and fishery activities in MRID mortality and their importance in different age classes of MRIDs (Beasley, 2007; CSG, 2012a; Dove, 2008; 2009; Gulland, 2009; 2014; Krützen et al., 2018; McLellan, 2010 pers. comm.; Reeves et al., 2009; Schnitzler et al., 2021; Smith, 2017; Thomas and Gulland, 2017; Vibol et al., 2009). Unfortunately, an endangered species recovery initiative has not been developed or implemented for the recovery of the MRID population. This is, in many ways, similar to the Baiji conservation initiative, which involved multiple meetings and workshops and much debate, without any tangible conservation outcomes (Turvey et al., 2007; Xu et al., 2008; Yang et al., 2006), culminating in the ultimate extinction of the species. For successful conservation recovery initiatives of endangered species/populations, such as the MRIDs, there needs to be early action, effective co-ordination and collaboration, reliable and sufficient funding and institutional capacity, together with an interdisciplinary skill set or team (Baillie, 2010). Gaskin (1982) stated that wildlife populations can show remarkable

resilience in the face of external pressures if their habitat is protected. Similarly, if conservation biology can be incorporated into policy development, then potentially population declines could be reversed, however sound scientific reasoning and balanced analysis and review is required to drive this approach. Ralls and Taylor (2000) stated "*it is better to err on the side of overprotection than under protection,* [then] *risk the extinction of a species*". This is the basis for conservation biology, and for the studies reported in this thesis.

Conservation biology addresses the biology of species, communities, and ecosystems that are influenced, either directly or indirectly, by human activities or other factors (Soulé, 1985). It can be a crisis discipline (Frankham, 2002), where it is not reasonable or practical to wait for prospective data collection before making decisions (Soulé, 1985). Thus, in a crisis discipline one must act before knowing all the facts, with the ultimate goal of preserving biological diversity (Soulé, 1985). In contrast, the Conservation Task Force focused on obtaining concrete evidence on why the population was declining in order to initiate a recovery strategy. This deviation from a conservation biology approach has delayed implementing a recovery initiative indefinitely. Over a decade on from the initial workshop, the MRID population continues to decline (Limsong et al., 2017), with grave consequences for the survival of the population. For example, the MRIDs in pool 9 were declared functionally extinct in 2016, with reports by WWF-Cambodia of only three of the seven dolphins remaining (Anonymous, 2016; Thomas and Gulland, 2017), and more recently media reports quoting Laos' PDR Department of Livestock and Fisheries officials stated that only one or two dolphins remained in this trans-boundary pool following the death of two dolphins in June 2021 (Asia News Network, 2021).

1.3 General overview of the study in this project

The focus of the studies reported in this thesis was to incorporate tools from several disciplines, namely conservation biology, conservation medicine, pathology, epidemiology, eco-toxicology and conservation genetics, to facilitate the investigation of the health status of the MRID population; and to determine if adverse health effects were contributing to the decline of the MRID population in Cambodia. This was deemed necessary as, despite extensive research on the MRIDs (Baird and Beasley, 2005; Beasley, 2007; Beasley et al., 2009; Beasley et al., 2013; Caballero et al., 2018a; Dove et al., 2007; Krützen et al., 2018; Schnitzler et al., 2021), there have been no quantitative analyses on the underlying causality driving the mortality in the population. As such, this collaborative study was initiated in February 2011, between Murdoch University, Universidad de los Andes, WWF (World Wildlife Fund for Nature)-Cambodia, Fundación Omacha, Sea Shepherd Conservation Society and The Palmari Natural Reserve.

The studies outlined in this thesis focus on the threats associated with mortality in the MRID population. This thesis is specifically concerned with the aetiology of mortality in different age classes of MRID, particularly adults and calves. Calves less than 110cm in length are defined in this study as neonatal calves (Beasley, 2007). Several aspects, potentially associated with mortality, were investigated in this study, including pathology and disease, immunosuppression, environmental contaminants and pollutants, and finally, genetic drivers, such as low genetic diversity and inbreeding/outbreeding depression.

The studies described in this thesis attempted to quantify the drivers of mortality by examining data collected over a ten year period. The health assessment studies on the MRID population reported in this thesis focused on three main possible drivers of mortality and

decline. Firstly, Schnitzler et al. (2021) documented several cases of disease pathology in the MRIDs, and suggested that immunosuppression may be a component in disease pathogenesis, similar to that observed by Bossart et al. (2003). As immunosuppression has been hypothesised to be the cause of skin lesions (Piccolo et al., 2016; Reif et al., 2009), a retrospective epidemiological study focusing on skin lesions in the MRID population was undertaken as one study in this thesis. Secondly, as several immuno-toxic contaminants, such as mercury (Hg), dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs), may result in immunosuppression (Schnitzler et al., 2021), a detailed toxicological study of tissue samples from deceased MRIDs formed the basis of another study reported in this thesis. Thirdly, limited genetic diversity has been identified in the MRIDs (Caballero et al. 2018a; Krützen et al. 2018), which, together with a high proportion of mortality in the population documented by Beasley (2007), may indicate that the population is ill-equipped to deal with environmental stochasticity. As such a genetic study was also undertaken using tissue samples collected from deceased MRIDs.

1.3.1 The aims of this study

This study was designed to determine:

1. The aetiologies of mortality and population decline in the MRID.

The specific objectives of the study were to:

1. Implement epidemiological tools and techniques to better evaluate health threats to the MRIDs.
2. To identify environmental stressors that may be contributing to the decline.

1.3.2 Hypothesis of this study

This thesis was designed to investigate the following research hypothesis:

Disease, together with interaction with fishery activities and genetic factors, is contributing to dolphin mortality and driving the MRID population decline and potentially the extirpation of the species from the Mekong River.

1.3.3 Overview of the chapters in this thesis

The organisation of the thesis and the studies conducted, along with the groups that I collaborated with for each of the studies, are outlined in Table 1-1. In order to address the research question the thesis contains six individual, yet interdependent, studies (Chapters 3 - 8). Through these six studies, I sought to answer specific research questions related to the complexities of disease investigation in the context of the mortality of the MRIDs. Summaries of all the dolphins included in this study are provided in Appendix 1: Tables 1 and 2.

Table 1-1: Outline of chapters in this PhD thesis

Chapter	Title	Objective	Research question	Collaborators
1	Introduction	Outlines the reason behind this study	What is the gap in knowledge of the mortality in the MRIDs?	
2	Literature review	Examines the literature on river dolphins, the causes of population declines, and the methodologies used to diagnose declining wildlife populations		
3	An epidemiological investigation of mortality in a population of Mekong River Irrawaddy dolphins over a 10-year period in Cambodia	Examines historical data from dead dolphins in the MRID population over a 10 year period to identify mortality trends	What are the drivers of mortality in the population, particularly in different age classes?	Dr. Martin Gilbert –WCS (Stranding database 2001-2006)
4	Pathobiological findings on necropsy examination of eight dead dolphins from 2007-2009, in the Mekong River, Cambodia	Examines eight necropsy cases to identify pathological aetiologies associated with mortality in five calves and three adults. Examining evidence in support of the disease hypothesis.	What evidence of disease pathology is there in deceased dolphins?	Prof. Thijs Kuiken- Erasmus University Medical Centre, the Netherlands (histological examination and interpretation)

Chapter	Title	Objective	Research question	Collaborators
5	Is necrotising fasciitis the cause of neck lesions in dead Mekong River Irrawaddy dolphins from Cambodia? A retrospective study	Examines 16 necropsy cases, 13 with neck lesions and three without neck lesions, that all had microbiological and cytological examinations to assess neck lesions in the dolphins and to identify the aetiology of neck lesions.	Is necrotising fasciitis responsible for the neck lesions found in the dead dolphins?	Dr Bertrand Guillard- Institut Pasteur du Cambodge (microbiology/cytology) Prof. Thijs Kuiken (histological examination and interpretation)
6	An epidemiological investigation of skin lesions in Mekong River Irrawaddy dolphins in Cambodia	This study reports on photo-identification examination of live MRIDs to assess if there is evidence of disease at the population level.	Are skin lesions prevalent in the population?	Dr. Fernando Trujillo- Fundación Omacha, Colombia (photo-identification research methodology) My research team of observers including: Dr. Fernando Trujillo, Troy Saville, Kim San Lor, Kim Sokha (WCS) Vin Bunna (MAFF), Tan Somethbunwath (MAFF), Loeu Theo (MAFF), Theng Lepin (MAFF) Bart Kluskens, Laura Parker, Richard Zanre, Raphael Woolf, David Dove, Gerard Ryan.

Chapter	Title	Objective	Research question	Collaborators
7	Toxicological analysis of deceased Mekong River Irrawaddy dolphins from Cambodia	This study examined the levels of environmental contaminants, particularly those considered immuno-toxic, in 22 deceased MRIDs to determine the level of these contaminants in this population.	What immuno-toxic contaminants are present in this population that may be contributing to mortality, immunosuppression, or disease?	Dr Ursula Siebert- Research and Technology Center Westcoast Christian-Albrechts-University Kiel, Germany and Dr Krishna Das- Laboratory for Oceanology – MARE Center, University of Liege, Belgium (toxicological review) Institute of Public Health in Ostrava and GALAB laboratories Gmbh (toxicological testing)
8	Multilocus analyses of the Mekong River Irrawaddy dolphins (Orcaella brevirostris) from Cambodia: insights into a recent population size decline	This study investigated the level of genetic diversity in the population, and examined genetic causes of high calf mortality, particularly inbreeding/outbreeding depression in the population, that may reduce reproductive success. In addition, this study assessed the paternity of calves in the population to ascertain the breeding potential of the females.	Are genetic factors contributing to the reproductive failure and population decline?	Prof. Susana Caballero – Universidad de los Andes (Supervision of genetics laboratory work, development of methodology) Dr. Paula Satizábal Universidad de los Andes (genetic data analysis)

Chapter	Title	Objective	Research question	Collaborators
9	General discussion	This chapter evaluated the data and key findings from the entire study and compares and contrasts that with the results of other studies to identify what is happening, what further research needs to be undertaken, and outlines what the limitations of this study were.		

1.4 Significance of this study

This study used an integrated, multidisciplinary approach involving ecologists, conservation biologists, conservation veterinarians, medical professionals, pathologists, microbiologists, toxicologists and geneticists, and the synthesis of technologies from all these diverse disciplines, as described by Daszak et al. (2001), to facilitate the understanding of mortality in the MRIDs. The proportion of dead dolphins with various threats/stressors was quantified as this is critical for determining whether or not the process of decline is reversible, or if the extirpation of these dolphins is inevitable, as has been suggested by Beasley et al. (2013), Krützen et al. (2018) and Caballero et al. (2018a). Diagnosing the cause of the high mortality in the MRID population and the population decline, is vital to designing mitigation strategies to improve recruitment and reversing the decline to ensure the survival of this unique population. Therefore, this study is of indisputable significance to the overarching conservation of this species.

1.5 Research approvals and ethics

The research in this thesis, using retrospective data and samples from cadavers, was approved by the Murdoch University Animal Ethics Committee. All fieldwork was approved by the Royal Government of Cambodia in collaboration with the DC and MAFF. Relevant CITES Permits were obtained (Cambodia Certificate no: KH 0669; Germany Certificate no: E-04406/09; and Colombia certificate no.:31595) for the export of samples from Cambodia to Germany and Colombia, respectively for further analyses.

CHAPTER TWO

Literature review

2.1 Introduction

River dolphins, including the MRIDs, are amongst the world's most endangered species of mammals (Braulik et al., 2015; da Silva et al., 2018; Veron et al., 2008; Williams, 2001). For decades, the threats to their survival received little attention in academic literature. However, this began to change in 2007, when one entire species, the Yangtze River dolphins known as the baiji, were thought to have become extinct (Ross et al., 2010). Since that time, it has emerged that river dolphins in Asia and South America (Table 2-1) (Williams, 2001) are also under threat.

River dolphins are found in Asia and South America and can be classed as either obligate (only found in freshwater) or facultative (found in major river systems as well as in estuaries and coastal marine waters). The obligate dolphins inhabit the river basins of the Amazon, Ganges, Indus and Yangtze rivers (Williams, 2001), and the facultative dolphins inhabit several major rivers and lakes as listed in Table 2-1. Obligate river dolphins comprise four distinct genera (Williams, 2001) which represent three ancient branches in cetacean evolution (Hamilton et al., 2001).

Table 2-1: Summary of freshwater cetaceans

Class	Species	Common name(s)	IUCN Red-list status	Distribution	References
Obligate species	*Lipotes vexillifer*	Yangtze River dolphin Baiji	Critically endangered*	Asia: (Yangtze and Qiantang Rivers, China)	Turvey et al., 2007
	Platanista gangetica	Ganges River dolphin	Endangered	Asia: (Ganges, Brahmaputra, Meghna, and Karnaphuli River systems)	Braulik et al., 2015; Braulik et al., 2021; Veron et al., 2008
	Platanista gangetica minor	Indus River dolphin	Endangered	Asia: (Indus River)	Braulik et al., 2021
	Inia geoffrensis	Amazon River dolphin Boto	Endangered	Central and northern South America: (Amazon, Madeira and Orinoco Basins)	da Silva et al., 2018
	Inia araguaiaensis	Araguaian boto	Not listed	Araguaia-Tocantins River system	Hrbek et al., 2014

Class	Taxon	Common name/s	IUCN Red-list status	Distribution	References
Facultative species	*Orcaella brevirostris*	Irrawaddy dolphin	Critically endangered	South East Asia: (Mekong River Cambodia, Laos PDR; Mahakam River/Semayang Lake system, Indonesia; Ayeyarwady River, Myanmar; Songkla Lake, Thailand; Chilika Lake, India)	Beasley, 2007; Beasley et al., 2009; Jefferson and Smith 2002; Kreb and Budiono, 2005; Stacey, 1996; Stacey and Leatherwood, 1997
	Sotalia fluviatilis	Tucuxi	Endangered	South America: (Amazon River Basin, Brazil; Colombia; Ecuador; Peru)	Veron et al., 2008
	Sotalia guianensis	Guiana dolphin	Near threatened	South America: (Orinoco River- Bolivia)	Caballero et al., 2007; Caballero et al., 2017; Cunha et al., 2005; Secchi, 2010
	Pontoporia blainvillii	Franciscana La Plata River dolphin	Vulnerable	Eastern coast of South America	Veron et al., 2008; Hamilton 2001; Hamilton et al., 2001
	Neophocaena phocaenoides	Finless porpoise	Vulnerable	Asia :(Yangtze River basin, Poyang and Dongting Lakes, China)	Jefferson and Smith 2002; Zhao et al., 2008; Zhao et al., 2013

* This species is now considered to be extinct (Turvey et al., 2007)

All river dolphins are severely threatened throughout their range due, primarily, to their close proximity to human settlements (Reeves et al., 2003; Smith and Reeves, 2012; Trujillo et al., 2010; Veron et al., 2008). As such they are much more exposed to anthropogenic activity than most oceanic dolphins, as they share the same habitat and compete for similar food resources as humans, making them more vulnerable to disturbance (da Silva et al., 2018) and direct mortality, as well as ecological disruption and environmental degradation that reduces the availability or quality of their freshwater habitat (Reeves et al., 2003; Smith and Reeves, 2012; Trujillo et al., 2010; Veron et al., 2008). Whilst the threats faced by river dolphins are similar to that in oceanic dolphins, the impacts may be magnified as river dolphins are restricted to their riverine habitat which is isolated and much smaller in comparison. The extinction of the baiji in the Yangtze River, China in 2006 (Turvey et al., 2007), highlights the potential grave consequences of such impacts (Marmontel et al., 2021) and as such will be discussed further in more detail.

Anthropogenic activities have resulted in the extinction of the baiji, the first dolphin species known to become extinct, and have also impacted the global biosphere and ecosystem, driving global extinctions of many other species of wildlife (Palumbi, 2001). As a result of anthropogenic activity we are in the period of the sixth great mass extinction, with biodiversity in decline and a current rate of extinctions estimated at 100-1000 times the pre-human “baseline normal” (Deem, 2011). This is due firstly, to the over-abundance of the human species (Hudson, 2003; Lacy, 1997). As the human population has expanded, it has encroached onto all lands of the planet, resulting in closer contact between wild animals, livestock and humans (Deem et al., 2000; Deem, 2011). This encroachment has resulted in the spread of disease to endangered wildlife species through contact with humans and domestic animals (Deem et al., 2000). Secondly, humans are capable of altering their

surroundings placing increasing pressure on the planet (Aguirre and Gomez, 2009), which has compromised the integrity of the global ecosystem (Pratarelli and Chiarelli, 2007). There are many similarities between the situation faced by the baiji, the global situation of mass wildlife extinctions, and the circumstances now confronting several dolphin species including the MRIDs (Beasley et al., 2013) and Indo-Pacific humpback dolphins (*Sousa chinensis*) (Ross et al., 2010), and porpoises such as the Yangtze finless porpoise (*Neophocaena phocaenoides*) (Huang et al., 2017; Mei et al., 2014) and the vaquita (*Phocoena sinus*) (Jaramillo-Legorreta et al., 2019). Thus, the extinction of the first dolphin species, the baiji, should be viewed as a global concern and a warning of the vulnerability of other dolphin populations, such as the MRIDs.

The risk of extinction/extirpation of wildlife species has been attributable either directly or indirectly to: climate change; emerging infectious diseases ('pathogen pollution'); biological impoverishment (e.g. loss of biodiversity and ecological processes such as habitat loss and degradation, and wildlife trade); and global 'toxification' (pollutants such as endocrine-disrupting chemicals) (Aguirre and Gomez, 2009; Deem, 2011; Hudson, 2003). These factors, in combination, are resulting in diminished human, domestic animal, wildlife and environmental health of the planet (Aguirre and Gomez, 2009).

This literature review follows a descriptive organisational structure, with the following intentions. Firstly, to identify threats specifically relevant to river dolphins. Secondly, I examined the literature on the baiji as an example of a river dolphin species that became extinct, to ascertain if there was anything that could be learnt and improved upon in hindsight to mitigate their extinction, that is applicable for the MRIDs which are critically endangered and at risk of extirpation (Beasley et al., 2013; Caballero et al., 2018a; Krützen et al., 2018).

Thirdly, I examined the literature to develop an investigational framework to explore mortality in the MRID population using principles from conservation medicine and conservation genetics, in order to structure the studies in this thesis and develop the health assessment methodology required for the MRIDs.

2.2 Threats to river dolphins

River dolphins are threatened by a multitude of stressors. Examples of direct mortality of river dolphins include: fisheries bycatch arising from entanglement in nets, electrocution through electro-fishing, or snagging on hooks (Gomez-Salazar et al., 2011; Kelkar and Dey, 2020; Kreb et al., 2010; Loch et al., 2009; Mansur et al., 2008; Paudel and Koprowski, 2020; Raby et al., 2011; Sinha, 2002; Veron et al., 2008); overexploitation and direct harvest of dolphins for their oil which is used as a fish attractant (Brum et al., 2015; Gomez-Salazar et al., 2011; Mintzer et al., 2020; Sinha, 2002) or for burning in lamps (Braulik et al., 2015); and disease (Bonar and Wagner, 2003; Paudel and Koprowski, 2020; Santos et al., 2011). In addition to direct threats, river dolphins are also threatened by: the development of hydroelectric and irrigation dams (Baruah et al., 2012; Brownell Jr et al., 2019; Braulik et al., 2015; Sonkar and Gaurav, 2020); environmental contamination (Barbosa et al., 2021; Garcia-Garin et al., 2021; Hung et al., 2006; Kannan et al., 2005; Kumari et al., 2002; Mosquera-Guerra et al., 2019; Schnitzler et al., 2021; Senthilkumar et al., 1999; Senthilkumar et al., 2001; Yeung et al., 2009); the loss or fragmentation of their habitat (Braulik et al., 2014; Braulik et al., 2015; Choudhury et al., 2019; Nabi et al., 2021; Sonkar and Gaurav, 2020); depletion of food resources by overfishing (Paschoalini et al., 2020; Paudel et al., 2020; Veron et al., 2008); incidental mortality from boat collisions; and disturbance or displacement by intensive vessel traffic or eco-tourism (Beasley et al., 2014; Veron et al., 2008). All these threats can act at the population level, singularly or synergistically, to drive decline in

abundance. Whilst some threats may appear obvious (e.g. bycatch and disease), others are more insidious (e.g. contaminants and genetics), with effects taking generations to be seen, and as such are often disregarded in their association with declining populations and mortality. These will all be reviewed further in this literature review.

2.2.1 Fisheries interaction/bycatch

Fisheries related mortality is considered to be the leading cause of death in river dolphins. This has been reported in Irrawaddy (Beasley, 2007; Brownell Jr et al., 2019), Ganges and Indus River dolphins (Dewhurst-Richman et al., 2020; Kelkar and Dey, 2020; Mansur et al., 2008), franciscana (Cunha et al., 2014), and in boto, tucuxi and Guiana dolphins (Iriarte, 2013). Brownell et al. (2019) considered bycatch to be a serious and pervasive threat, hindering conservation efforts of endangered small cetacean species. For instance, fisheries related mortality was thought to be the ultimate driver behind the extinction of the baiji (Turvey et al., 2007) and is believed to be the principal cause of mortality in the MRIDs by the conservation task force (Gulland, 2009; 2014 ; Reeves et al., 2009).

2.2.2 Disease

Disease in river dolphins has only been sporadically reported with reports of skin lesions in Ganges River dolphins (Paudel and Koprowski, 2020), toxoplasmosis (Santos et al., 2011) and golf ball disease associated with *Streptococcus iniae* (Bonar and Wagner, 2003) in Amazon River dolphins, verminous pneumonia from *Halocercus brasiliensis* and bacterial pneumonia in both Amazon River dolphins and tucuxis (Rodrigues et al., 2018) and morbillivirus in Guiana dolphins (Flach et al., 2019).

Disease can have both direct and indirect effects on a population's size, and has been linked to extinction of wildlife species (Gerber et al., 2005; McCallum and Dobson, 1995; Pedersen et al., 2007; Soulé, 2002), including mammals such as the thylacine (*Thylacinus cynocephalus*), the Galapagos mouse (*Nesoryzomys darwini*) and the indefatigable Galapagos mouse (*N. indefessus*), as well as many frog species (Smith et al., 2006). However, previously disease has not been linked to the extinction of the baiji, nor to the declining numbers of river dolphins. Unfortunately, disease investigations and disease surveillance programs are not routinely conducted in river dolphin species, with the exception of the mortality investigation program on the MRIDs, discussed in more detail in this thesis.

2.2.2.1 Emerging infectious diseases (EIDs)

Emerging infectious diseases (EIDs) can be defined as infections that have either recently appeared in a population, or have existed previously but are rapidly increasing in incidence or geographical range (Morse, 1995). There is a paucity of reports in the literature on EIDs in river dolphins, however EIDs have been described in oceanic dolphins, for example poxvirus infection ('Tattoo Skin Disease') in Striped Dolphins (*Stenella coeruleoalba*) (Cocumelli et al., 2017), novel dolphin morbillivirus also in *S. coeruleoalba* (Di Guardo and Mazzariol, 2016; Pautasso et al., 2019) and skin nodules in *O. brevirostris* (Van Bressem et al., 2014).

Emerging diseases may have a complex causality involving multiple co-factors, such as anthropogenic contaminants, genetics, and immunologic dysfunction such as immunosuppression. Furthermore, they may also have zoonotic implications and epizootic potential (Bossart, 2011). Several factors have been described that facilitate the emergence of EIDs in wildlife: ecosystem alteration from anthropogenic or natural events; movement of

pathogens or vectors; and changes in pathogen virulence, pathogenicity and infectivity from genetic drift. Additionally, advances in epidemiology has resulted in increased reporting, more accurate (sensitive) tests and an increased number of studies which have resulted in detection of diseases that may have been present in the populations for some time (Williams et al., 2002). Over the past two decades there has been a better understanding of the link between the environment and disease, with many infectious and non-infectious diseases having ecologic drivers (Daszak et al., 2006; Naish et al., 2014). Understanding ecological linkages and complex causality with multiple co-factors may be a crucial part of a mortality investigation of the MRIDs.

2.2.3 Chemical pollution

Dolphins are apex predators/carnivores (Bossart, 2011; Moeller, 2003), and as such they are exposed to a variety of persistent organic pollutants (POPs) and inorganic pollutants that bioaccumulate in aquatic ecosystems (Bossart, 2011). Dolphins are capable of storing a range of synthetic organochlorine contaminants, methylmercury, organotins and other industrial chemicals at concentrations which have been reported to result in adverse effects in other animals and humans (Colborn and Smolen, 2003). For example, the presence of certain POPs in dolphins have been linked to increased susceptibility to infectious diseases (Hall et al., 2006; Jepson et al., 2005; Ross et al., 1996; Ross, 2002), immunosuppression (Béland et al., 1991; Colborn and Smolen, 2003; de Guise et al., 1998; de Swart et al., 1994; Mori et al., 2008; Pellisso et al., 2008; Ross et al., 1996), reproductive impairment (Colborn and Smolen, 2003; Jefferson et al., 2006; Jepson et al., 2005; Law, 1996; Reijnders, 1986; Schwacke et al., 2002), endocrine disruption (Brouwer et al., 1999; Colborn, 2004; Yordy et al., 2010a), and neoplasia (Colborn and Smolen, 2003; Martineau et al., 1994; Martineau et al., 1988).

Although these studies highlight that exposure to environmental contaminants have potential negative implications on dolphin populations (O'Shea et al., 2003), few studies have demonstrated a causal association.

Chemical pollution is recognised as a global problem affecting wildlife and human health (Colborn, 2002). Industrial and organochlorine chemicals, such as plastics, petroleum derivatives and pesticides, are known sources of environmental contamination (Mangini et al., 2012). Some chemicals, such as pesticides, can produce acute toxicity (e.g., neurological effects, skin lesions and death), whilst others can produce more insidious, non-lethal effects to animal health (Mangini et al., 2012). For example, endocrine disruptors (EDCs) affect reproduction, foetal development and maturation, senescence, immunological responses and behaviour by interfering with the hormonal control of several metabolic processes (Colborn, 2002).

2.2.3.1 Timeline of chemical pollution

Since the mid-1940s, the chemical composition of the biosphere has changed due to the release of large quantities of synthetic chemical (xenobiotic) pollutants into the environment, particularly the world's waterways and oceans (Carvan III and Busbee, 2003; Colborn and Smolen, 2003). These pollutants include chemicals from industrial accidents and leaching from toxic dumps, as well as pesticides from agricultural run-off and atmospheric drift (Carvan III and Busbee, 2003). Such chemicals include the polychlorinated biphenyls (PCBs) which were introduced in 1929, Dichlorodiphenyl-trichloroethane,1,1,1-trichloro-2,2-*bis*[*p*-chlorophenyl]ethane (DDT) which became available for retail sale in 1938 and from 1942 was used worldwide to combat the mosquito vector of malaria, and since the late 1940s a

large number of synthetic chemicals, including industrial, and agricultural organochlorines including dieldrin, chlordane, toxaphene, hexachlorocyclohexane (HCH), and Hexachlorobenzene (HCB) (Colborn, 2004; Torres-Sánchez et al., 2007). The production and use of industrial and agricultural chemicals has increased exponentially since the 1950's (Colborn, 2004). However, in the 1970's several organochlorines (OCs), together with DDT and PCBs, were banned in a number of developed countries, due to their persistence and long-half life in the environment (Colborn and Smolen, 2003). However, these chemicals were still widely used in Cambodia when this study commenced (EJF, 2002; Jensen et al., 2011; Minh et al., 2006; Wang et al., 2011) and in the neighbouring countries of Vietnam (Nguyen et al., 2019; Wang et al., 2016), Thailand (Poolpak et al., 2008; Sudaryanto et al., 2011; Tanabe et al., 2000), and Laos PDR (Sudaryanto et al., 2011). Since the early 2000s, the focus of chemical pollution in dolphins has shifted towards "emerging" unregulated contaminants, including pharmaceuticals and personal care products, hormones, UV filters, perfluorinated compounds, polybrominated flame retardants (BFRs), pesticides, plasticisers, artificial sweeteners, illicit drugs, and EDCs (Adams et al., 2014; Nollet and Lambropoulou, 2014). Microplastics (< 5mm) have also been reported in dolphins in recent years (Hernandez-Gonzalez et al., 2018; Novillo et al., 2020; Pfeifer, 2020; Zhang et al., 2021), however the long-term consequences arising from their ingestion are not known (Pfeifer, 2020), and their impact on dolphins requires further investigation.

2.2.3.2 Contaminants in river dolphins

Although there are few studies on river dolphins compared with oceanic dolphins, several studies have reported the levels of contaminants in river dolphins (Table 2-2).

Table 2-2: Summary of contaminant studies in freshwater cetaceans

Dolphin species	Contaminants*	Reference
Irrawaddy dolphin (Chilika Lake)	OCs, PCBs, DDTs, PBDEs, HCHs	Kannan et al., 2005
Irrawaddy dolphin (Mekong River, Cambodia)	PCBs, DDTs, PBDEs, Dioxins, Mercury	Dove, 2009
	OCs, PCBs, DDTs, Dioxins and furans (PCDD, PCDF), BFRs-PBDEs, PFOS, PFOA, OTCs,	Schnitzler et al., 2021
	Heavy metals (As, Pb, Cd, Fe, Cu, Hg, MeHg, Se, Zn)	Schnitzler et al., 2021
Ganges River dolphin	PCBs	Senthilkumar et al., 1999; Senthilkumar et al., 2001
	OCs	Hung et al., 2006; Kumari et al., 2002; Senthilkumar et al., 1999
	Perfluorinated compounds	Yeung et al., 2009
	PCDDs, PCDFs	Senthilkumar et al., 2001
	Butylin	Kannan et al., 1997
Botos	Heavy metals (Hg, MeHg)	Barbosa et al., 2021; Mosquera-Guerra et al., 2019
Franciscana dolphins	Heavy metals	Garcia-Garin et al., 2021
Finless porpoise	PCBs	Senthilkumar et al., 1999

*Organochlorine pesticides (OCs), Polychlorinated biphenyls (PCBs), Dichlordiphenyltrichlorethans (DDTs), Hexachlorocyclohexanes (HCHs), Brominated flame retardants (BFR): polybrominated diphenyl ethers (PBDEs), polyfluoroalkyl substances (PFOS, PFOA), organotin compounds (OTCs), arsenic (As), lead (Pb), cadmium (Cd), Iron (Fe), copper (Cu), mercury (Hg), methylmercury (MeHg), selenium (Se) and zinc (Zn).

2.2.3.3 Persistent organic pollutants (POPs)

The presence of persistent organic pollutants (POPs) have been widely reported in dolphins (Alonso et al., 2012). These POPs include a wide range of halogenated aromatic hydrocarbons (HAHs), such as the halogenated-biphenyls (PCBs), dibenzofurans (PCDFs), and dibenzo-*p*-dioxins (PCDDs), the most toxic of which is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and DDT and its associated metabolites (Carvan III and Busbee, 2003). Many of the POPs found in dolphins are considered to be EDCs. These are defined by the United States Environmental Protection Agency (EPA) as "an exogenous chemical substance or mixture that alters the structure or function(s) of the endocrine system and causes adverse effects....at the level of the organism, its progeny, and populations or subpopulations of organisms" (Schneider et al., 2003). These interact with steroid receptors in cells, thereby altering hormone metabolism, serum hormone levels and hormone regulated gene expression (Carvan III and Busbee, 2003). Example of EDCs detected in dolphins include: pesticides, such as synthetic pyrethrins and DDT; brominated flame retardants (polybrominated diphenyl ethers - PBDEs); herbicides (atrazine, glyphosate); bisphenol-A (BPA) used to make plastics harder, clearer, and more resistant to heat stress; and phthalates for plastic pliability (Alonso et al., 2012; Schneider et al., 2003).

The impact of EDCs in wildlife has been well documented (Brouwer et al., 1999, Colborn, 2002; Colborn, 2004; Colborn and Carroll, 2007; Dórea, 2008; Dzieweczynski, 2011; Patisaul and Adewale, 2009; Schwacke et al., 2012; Torres-Sánchez et al., 2007). Despite this, few studies exist on the physiological effects of chronic exposure to sub-acute levels of the EDCs (Carvan III and Busbee, 2003; Colborn, 2002; 2004; Patisaul and Adewale, 2009), although there is a growing body of research documenting the impact of EDC on dolphins

(Hart et al., 2020; Homer-Drummond, 2012). Yordy et al. (2010a) believed that chronic exposure to EDCs could have a detrimental impact on dolphin populations, as EDCs are able to mimic or interfere with endogenous hormones and other signalling chemicals of the endocrine system (Colborn, 2004). Specifically, EDCs can: impair reproductive fitness and cause premature reproductive senescence, reducing the ability to produce viable, fertile offspring (Homer-Drummond, 2012; Schneider et al., 2003); impair testosterone production in male dolphins (Trego et al., 2018); disrupt lactation in females (Schneider et al., 2003); and have been found to alter behaviour in other species, such as the three-spined stickleback fish (*Gasterosteus aculeatus*) (Dzieweczynski, 2011) and Sprague-Dawley rats (Krishnan et al., 2019). This altered behaviour can be sex specific (Schneider et al., 2003), and includes increased aggression in males towards conspecifics and/or offspring. These changes highlight the need to observe successive age classes of dolphin populations closely for: behavioural changes; susceptibility to disease; and changes in reproductive success and population age structure (Colborn and Smolen, 2003).

Bioaccumulated HAHs in dolphins were previously assumed to be inert (Carvan III and Busbee, 2003), however they can potentiate disease through altering gene expression (Carvan III and Busbee, 2003; Choi et al., 2004; Leijs et al., 2019; Noël et al., 2014) resulting in a range of adverse effects in dolphins. These can include immunotoxicity (Fair et al., 2021), neurotoxicity (Pessah et al., 2019), hepatotoxicity (Sonne et al., 2005), reproductive dysfunction/developmental toxicity (Murphy et al., 2015), foetotoxicity and carcinogenicity (Carvan III and Busbee, 2003; Choi et al., 2004).

POPs are lipophilic and hence the primary storage depot for POPs in dolphins is the blubber (Bossart, 2011; Mössner and Ballschmiter, 1997), which contains 90% of the whole-body

contaminant burden (Yordy et al., 2010b). Persistent organic pollutants bioaccumulate through the foodchain (Alonso et al., 2012; Bossart, 2011; Carvan III and Busbee, 2003; Jensen et al., 2011), and therefore although environmental concentrations of POPs may be low, bioaccumulation can result in high levels and associated detrimental impacts on health of dolphins (Alonso et al., 2012; Carvan III and Busbee, 2003; Moeller, 2003).

Bossart (2011) found that blubber followed a dynamic process, with lipid deposition occurring when dietary intake exceeded metabolic demand, and conversely mobilisation of lipids from fat reserves occurred when energy was needed to meet the demands associated with thermoregulation, reproduction, lactation, and disease or nutritional/energy deficit. Thus, during periods of decreased food intake, lactation, or other physiological demands, bioaccumulated contaminants in the blubber may be mobilised and redistributed into the circulation (Bossart, 2011). This redistribution may then affect adult and peri-natal health (Bossart, 2011), particularly in individuals with reduced blubber-lipid stores, as they mobilise their fat stores more readily (Yordy et al., 2010b).

2.2.3.4 Mercury

Mercury (Hg) has been reported as a concern in the MRIDs by Dove (2009) and more recently by Schnitzler et al. (2021) who commented that the high percentage of organic Hg found in the MRIDs was unusual, although they could not provide an explanation for the elevated level.

Exposure of dolphins to Hg primarily arises from natural sources and anthropogenic exposure (Colborn and Smolen, 2003), for example gold mines that use Hg to amalgamate the

extracted gold. Numerous studies have documented total Hg levels, which includes metallic mercury and organic and inorganic compounds (Colborn and Smolen, 2003), in river dolphins (Table 2-2) and also in oceanic dolphins, including striped dolphins (*S. coeruleoalba*) and bottlenose dolphins (*Tursiops truncatus*) (Bellante et al., 2017; Bennett et al., 2001; Bernier et al., 1996; Bussolaro et al., 2010; de Moura et al., 2012; Gui et al., 2014; Mollenhauer et al., 2009; Mosquera-Guerra et al., 2019). Most metallic Hg is passed through the body without being absorbed (Colborn and Smolen, 2003). In contrast, the highly toxic organic compound methylmercury (MeHg) is fat soluble and is thus stored in animal tissue through biomagnification, and moves up the food chain to the apex predators, in this case the dolphins (Bossart, 2011; Colborn and Smolen, 2003; de Guise et al., 2003). Colborn and Smolen (2003) found that MeHg readily crosses the placenta, sequestering in the foetal brain, and is considered a developmental neurotoxin, which has potential severe implications for dolphin calves.

2.2.3.5 Contaminants, immunosuppression and disease

The reduction in the population numbers of many marine mammal species has raised concern about the potential role of environmental contaminants in these declines (Moeller, 2003). Xenobiotics of major concern due to their immunotoxicity include: organochlorins, such as PCB's, dibenzofurans (PCDFs), dibenzodioxins (PCDDs); organochlorine pesticides (DDT, toxaphene and others); and heavy metals (Hg, Pb, and Cd) (Moeller, 2003). The excessive accumulation of these immuno-toxic xenobiotics in dolphin calves from *in-utero* transfer (Aguilar and Borrell, 1994; Borrell et al., 1996; de Moura et al., 2008) and lactational transfer (Law, 1996; Ridgway and Reddy, 1995; Rosas and Lehti, 1996; Puchkova et al., 2018), as well as in older animals, can result in thymic atrophy, pancytopaenia and contaminant-

induced immunosuppression, leading to underlying health problems and epizootics; poor health; increased susceptibility to infectious agents; and tumour development (Brousseau et al., 2003; de Guise et al., 2003; Moeller, 2003; Mössner and Ballschmiter, 1997; Yordy et al., 2010a). Thus, it is important to evaluate dead dolphins for immuno-toxic contaminants which may be causing immunosuppression and increasing the risk of infection with opportunistic pathogens (Yordy et al., 2010a). For example, Moeller (2003) found that bacteria that are part of the normal flora of a dolphin, such as *Edwardsiella*, *Klebsiella*, or *Streptococcus* spp., can gain entry to the vascular system through traumatic injuries, resulting in rapid haematogenous spread to other organs causing infections and death. The St. Lawrence (SLE) population of beluga whales (*Delphinapterus leucas*) are reported to be immunosuppressed due to exposure to high levels of immuno-toxic contaminants, resulting in severe infections with bacteria that usually have a low pathogenicity (Brousseau et al., 2003; de Guise et al., 1999; de Guise et al., 1995; Desforges et al., 2012; Martineau et al., 1994; Martineau et al., 1988; Martineau et al., 2003; Muir et al., 1990; Ruby et al., 2003). Furthermore, toxins (particularly xenobiotics) can lead to immunosuppression and increased susceptibility to other infectious agents, such as viruses, which can result in further immunosuppression and/or the loss of integrity of the host barriers (Beineke et al., 2010; Moeller, 2003). For example, animals, including mammals, birds and fish, exposed to PCBs naturally or experimentally are more susceptible to viral infections (de Guise et al., 2003; Waugh et al., 2018). In one study, striped dolphins (*S. coeruleoalba*) infected with morbillivirus during the 1990 morbillivirus epizootic were shown to have elevated levels of PCBs, compared to striped dolphins previously biopsied in the same area, prior to the outbreak (Aguilar and Borrell, 1994).

2.2.4 Genetic threats

Population extinctions/extirpations occur as a result of the combination of deterministic and stochastic factors (Shaffer, 1981). Stochastic factors include: environmental uncertainty (unpredictable or random variations in disease, parasites, food supply, habitat quality, climate change, predators, competitors); demographic uncertainty (random variations in survival and reproduction success, birth rates, mortality rates and sex ratios of members of a population); and genetic uncertainty (genetic drift, inbreeding/outbreeding depression, loss of genetic variation/diversity, mutation accumulation) (Brook et al., 2002; Frankham et al., 2002; Rosel and Reeves, 2000; Shaffer, 1981). In contrast, deterministic factors reflect what is known about population responses to environmental drivers, and can be identified by relating environmental drivers to a reduction in population size (Benedetti-Cecchi et al., 2015) for example habitat degradation, pollution, and overfishing (Frankham, 1995a).

Genetic factors affect extinction/extirpation risk because most threatened species have small or declining numbers, and in such circumstances inbreeding and loss of genetic diversity are unavoidable (Frankham et al., 2002; Spielman et al., 2004). Processes that can have large effects on small populations include: genetic drift, inbreeding, bottlenecks and founder effects which further reduce genetic variability (Rosel and Reeves, 2000) as demonstrated by Zhivotovsky et al. (2015) in Atlantic cod (*Gadus morhua kildinensis Derjugin*). The MRID population is a small declining population (<100 individuals) comprising only 5% of their historical population (Krützen et al., 2018), thus it is certain that they are impacted genetically. For instance, small populations lose genetic variation as a consequence of genetic drift and at the same time become inbred, resulting in reduced fitness (Reed and Frankham, 2003). The loss of genetic diversity in small populations increases extinction/extirpation risk by adversely affecting the ability of populations to evolve and adapt to environmental

changes (Frankham, 2005). Small populations are extremely vulnerable to random fluctuations in reproductive success of breeding adults, and as a result, the genetic consequences can be severe (Rosel and Reeves, 2000). These small isolated populations can suffer from inbreeding, with increased homozygosity and higher relatedness between individuals than in larger populations (Pertoldi et al., 2007). This usually leads to the loss of genetic variation, especially if the populations remain small for long periods (NRC, 1995). Small populations are also sensitive to stochastic changes, and reduced genetic variation results in lower fitness, a reduced ability to adapt to changes in their environment over time and decreased evolutionary potential culminating in extinction/extirpation (Pratarelli and Chiarelli, 2007; Rosel and Reeves, 2000). This is particularly important for the MRIDs, as they have a restricted geographical distribution and specialised habitats, which limits their ability to move to new locations if their environment is subjected to degradation or destruction (Rosel and Reeves, 2000). As such, they are dependent on adaptive change to cope with environmental changes, making genetic variability an important conservation concern (Rosel and Reeves, 2000). Furthermore, if members of the population do not mate with others at random (the case for most natural populations) then the effect of the small population size on loss of genetic variation is further exacerbated. In this situation the population is said to have a smaller effective size (N_e) than its true size (N) (NRC, 1995). Rosel and Reeves (2000) provided an example that is relevant to the MRIDs. They demonstrated that, although there may be 100 dolphins in a segment of river, the number contributing to the genetic diversity of the next generation (N_e) could be as few as 25 individuals, representing a critically low number that may raise concern about the loss of genetic variability in such a small population. As such it is important to determine how many individuals in the MRIDs are actually contributing to the genetic diversity of the next generation through the use of paternity testing.

2.2.4.1 Genetic drift in small populations

In many endangered species genetic drift is important, either because of a currently small population size or because of past severe bottlenecks or founder events (Hendrick and Kalinowski, 2000). In small populations, genetic drift can cause fixation of detrimental alleles, resulting in most individuals having lower fitness compared to other populations (Hendrick and Kalinowski, 2000). Therefore, genetic drift causes more dramatic changes in gene frequency in small populations than in large populations. Rosel and Reeves (2000) found that as heterozygosity was lost with each successive generation, a population of 50 individuals would lose 82% of their genetic variability over 25 generations. In dolphins, which are long lived animals, these changes will take many years to show up; however the effects of genetic drift may be exacerbated by inbreeding or outbreeding (Rosel and Reeves, 2000).

2.2.4.2 Population bottlenecks

The bottleneck effect was defined by Chakraborty and Kimmel (2001) in the context of conservation genetics as the consequences of a temporary reduction (and subsequent expansion) of population size on genetic variation. Population bottleneck effects may be induced by disease epidemics, indiscriminate killing, and geographical isolation, such as from dam construction (Chakraborty and Kimmel, 2001). Similarly, ‘founder effect’ has been defined as the genetic composition of a population initially formed by a number of founder individuals (Chakraborty and Kimmel, 2001). Krützen et al. (2018) suggested that the MRIDs were subjected to a founder effect.

There are several profound consequences of a bottleneck population. Firstly, individuals in a bottlenecked population remain inbred relative to randomly selected individuals in the source population (Fowler and Whitlock, 1999). Secondly, prolonged population bottlenecks will lead to greater loss of variation through genetic drift (Lacy, 1997). Thirdly, populations that have lost genetic variability during a bottleneck event will have an excess of loci with an abundant heterozygosity, which can be used to identify such populations (Luikart and Cornuet, 1998; Luikart et al., 1998; Spencer et al., 2000; Waldick et al., 2002). As a result of these consequences, a population will have reduced fitness and suffer inbreeding (Lacy, 1997).

2.2.4.3 Inbreeding/outbreeding depression and extinction risk in small populations

Inbreeding (the mating of closely related individuals) differs from inbreeding depression (the reduced fitness of the offspring of these matings, compared to the offspring of randomly mated individuals) (Hendrick and Kalinowski, 2000; Lacy, 1997). Similarly, outbreeding between diverged populations may also lead to reduced reproductive fitness, known as outbreeding depression (Edmands, 2007; Frankham et al., 2002; Houde et al., 2011; Marr et al., 2002; Sagvik et al., 2005). Inbreeding, has deleterious consequences on all aspects of reproduction and survival (Frankham, 2005), with both inbred individuals and inbreeding depression reducing the fitness of inbred individuals relative to outbred individuals (Darwin, 1876; Fowler and Whitlock, 1999). There is now compelling evidence that all species show some level of inbreeding depression (Lacy, 1997; O'Grady et al., 2006), including wild species in their natural habitats (Frankham, 2005).

The link between inbreeding and extinction/extirpation has been well documented (Brook et al., 2002; Finke and Jetschke, 1999; Frankham, 1995b; 1998; 2001; Frankham et al., 2001; Frankham and Ralls, 1998; Fredrickson, 2007; Lucy et al., 2008; O'Grady et al., 2006; Reed et al., 2003a; Reed et al., 2002; Saccheri et al., 1998; Wright et al., 2008). In particular, extinction/extirpation of small populations is extrinsically linked to both inbreeding and outbreeding depression. A challenge exists however, in obtaining a reliable assessment of the contribution of inbreeding and outbreeding depression to the risk of population extinction/extirpation (O'Grady et al., 2006; Pertoldi et al., 2007). Frankham (1995b) found that the rates of extinction remained low through early generations of inbreeding until a threshold was reached following several generations of inbreeding, and then extinction rates increased dramatically. Similarly, Houde et al. (2011) found that inbreeding for one generation and outbreeding for two generations may have similar effects on small populations, leading to extinction/extirpation. Brook et al. (2002) used a computer model to compare the median time to extinction for the baiji, with and without inbreeding, for an initial population of 50, 250 or 1000 individuals. They found that the time to extinction was 65, 107 and 146 years respectively, and that inbreeding only decreased this time by 2%, 5% and 2%, respectively. In a small population, the genetic load, represented by potentially deleterious recessive alleles (Lacy, 1997; Pertoldi et al., 2007) that become fixed from genetic drift during population bottlenecks (Lacy, 1997), may increase the probability of extinction/extirpation (Frankham, 1998; Fredrickson, 2007; Lucy et al., 2008; Reed et al., 2003a; Saccheri et al., 1998; Samuel and Bruno, 2002; Wright et al., 2008; Zschokke and Baur, 2002). For example, Slatis (1960) found that the deleterious effects of inbreeding depression in a post-bottleneck herd of European bison (*Bison bonasus*) were largely due to the presence of recessive lethal genes, rather than a lack of heterozygosity. Yet, despite significant heterozygosity in these bison, inbreeding depression still occurred, with infant

mortality increasing as the population became more inbred (Lacy, 1993). Spielman et al. (2004) also demonstrated that 77% of threatened taxa exhibited reduced genetic diversity. However, declining populations may have similar genetic diversity to taxonomically related non-threatened taxa because they are less affected by inbreeding, and are driven to extinction/extirpation so rapidly that genetic factors have insufficient time to impact them (Frankham, 2005; Lande, 1988).

2.2.4.4 Minimum viable population size

It is not possible to establish a universally applicable minimum viable population size (Rosel and Reeves, 2000); however small populations (<100 individuals) are considered extremely vulnerable to the accumulation of deleterious genetic mutations (Lynch, 1996). Furthermore, O'Grady et al. (2006) found that inbreeding depression is a major threat for most mammalian taxa with small population sizes comprising <1000 individuals. Franklin (1980) suggested that to avoid the deleterious effects of inbreeding, the minimum population size should be 50 individuals to retain reproductive fitness, as below this number the risk of extinction would be accelerated. However, Franklin stated that to maintain sufficient genetic variability for adaptation to changing environmental conditions, the minimum N_e should be around 500.

2.2.4.5 Genetic conservation implications

An extinction/extirpation vortex is one of the greatest threats to endangered species. This occurs when demographic, environmental and genetic stochastic factors interact with each other and with deterministic factors to reinforce the demise of a small population (Johnson, 2010). Cumulative changes from genetic drift, inbreeding and outbreeding in small populations pose challenges to conserving species in nature (Altizer et al., 2003). As such,

conservation strategies need to consider the rate at which a threatened population becomes inbred (Pertoldi et al., 2007), as if inbreeding occurs suddenly then genetic drift plays a major role (Day et al., 2003), whereas the purging of mildly deleterious alleles only occurs when inbreeding happens gradually over several generations (Reed et al., 2003). Thus, the role of inbreeding/outbreeding depression in the extinction/extirpation risk of wild populations needs to be factored in, as disregarding them will result: firstly, in serious overestimates of the survival prospects of threatened species; and secondly, in the implementation of inappropriate recovery plans if the causes of extinction/extirpation risk and their relative contributions are not recognised (O'Grady et al., 2006). Ideally, all processes that affect endangered populations should be comprehensively assessed and incorporated into conservation plans to enable species recovery and mitigate an extinction vortex (Johnson, 2010).

2.3 Extinction of the baiji: learning from history

The baiji (*L. vexillifer*) was previously endemic to the Yangtze River, China (Reeves and Gales, 2006; Wang et al., 2006) and represented a clade that diverged from other cetacean lineages more than 50 million years ago (Baillie, 2010; Nikaido et al., 2001). In 2006 the likely extinction of the baiji was declared, following a six-week survey that failed to find any evidence of the species (Turvey et al., 2007). This extinction has been described by Reeves and Gales (2006) as "the equivalent to snapping off a complete evolutionary branch from the tree of mammalian radiation" and was considered avoidable (Baillie, 2010).

The extinction of the baiji was significant in two respects. Firstly, the baiji is the first cetacean species known to have been driven to extinction by anthropogenic impacts (Jaramillo-Legorreta et al., 2007; Turvey et al., 2007). These impacts included: incidental mortality from fisheries interaction; loss or degradation of habitat by construction of dams

and riverine development; agricultural and industrial pollution; and water vessel traffic (Reeves and Gales, 2006; Smith et al., 2008; Yang et al., 2006). This is significant, as other cetaceans, such as the MRIDs and the vaquita (*P. sinus*), are also edging closer towards extinction due to anthropogenic causes (Beasley et al., 2013; Jaramillo-Legorreta et al., 2007). Secondly, the extinction of the baiji represents the first documented mammalian species lost since the emergence of an international network of conservation organisations that prioritised conservation efforts on charismatic vertebrates (Turvey et al., 2007). Whilst these conservation task forces are well intentioned, they also appear to hinder conservation efforts. For instance, an *ex situ* reserve called Tian-e-Zhou (Shishou Oxbow Reserve) was developed in 1992 by the Chinese government as a final option to save the baiji. This reserve was to provide a safe place to support a breeding population of baiji, as had been successfully demonstrated in a feasibility study using the finless porpoise (Zhang et al., 1995), even though the long-term success of the finless porpoise was considered questionable due to inbreeding depression (Huang et al., 2017). Although a conservation recovery action plan to save the baiji was approved by the Chinese government in 2001, the international workshop to design a recovery strategy did not occur until 2004 (Xu et al., 2009; Yang et al., 2006). This workshop focused on strategies for capture, translocation, and *ex situ* management of the remaining baiji from the Yangtze River as a final attempt to save the species from extinction, which was believed to be inevitable at that time if no remedial action was taken (Yang et al., 2006). Ultimately, whilst well-intentioned, the conservation task force did little to enact the conservation action plan, nor to develop and implement a recovery strategy for the baiji. Thus, despite extensive scientific debate and several workshops (Wang et al., 2006) spanning two decades (discussed in more detail below), little effort was ever made to actually implement the *ex situ* baiji recovery programme (Turvey et al., 2007). The only baiji captured and released in the Shishou Oxbow Reserve was a female captured in 1995, however less

than seven months later she was found entangled in the escape-prevention net at the outlet of the reserve (Dudgeon, 2005; Smith et al., 2008). Turvey (2008) wrote a book on how we failed to save the baiji from extinction, where the need for direct action to implement a recovery plan is discussed, as had been done previously for other species, not just discuss and debate it for 30 years, as ended up being the case for the baiji. Similarly, Jaramillo-Legorreta et al. (2007) reported that "scientists must play a critical role in working to inform and assess conservation planning", and the extinction of the baiji represents a failure of scientists to explain simplistically the plight of a critically endangered species, thus failing decision makers that needed to act decisively and urgently. In the case of the baiji, Turvey (2008) emphasised the need for one conservation body or leader to take control, to fund raise, and to implement the strategies proposed by the conservationists, particularly the *ex situ* recovery program. Turvey believed that had this been achieved, the baiji would have had a fighting chance of survival.

2.3.1 The baiji and *ex situ* conservation: the great debate

Ex situ conservation for the baiji was a controversial proposal, with Dudgeon (2005) reporting concerns by international agencies over the risks associated with such an initiative. Initially, Yang et al. (2006) opposed the *ex situ* conservation strategy, instead strongly advocating ecological triage. They argued that the scientific community should use the available evidence to advise on the redirection of resources towards species and habitats with the highest likelihood of persistence, and whose conservation may actually have a successful outcome. Reeves and Gales (2006) disagreed with this, stating that, although the evidence available gave a grave prognosis and offered no basis for optimism about the baiji's viability in nature, they supported the decision for an *ex situ* recovery plan based on a similar plan

implemented successfully with the Yangtze finless porpoise (Mei et al., 2014; Wang et al., 2009). They did not agree that an aggressive effort to establish a small population of baiji in the reserve would be a major setback to biodiversity conservation as was reported by Yang et al. (2006). Reeves and Gales (2006) agreed that, although such a recovery strategy posed substantial risks, they advocated that it should not be dismissed on the basis of historical failures. Wang et al. (2006) insisted that in order to maximise future options and provide insurance against extinction it was essential to establish a population by preserving at least some of the baiji in the *ex situ* reserve. Despite the ongoing debate about the plausibility of this recovery initiative, Wang et al. (2006) believed that there was only a slim chance of success in collecting enough baiji to develop a viable *ex situ* program within the reserve.

Yang et al. (2006) argued that conservation measures for recovery of the baiji occurred too late in the process, when numbers were already so low that the probability of success was unlikely. This was further supported by Smith et al. (2008) who stated that the expectation that sufficient numbers of baiji could be captured and placed in a reserve to establish an *ex situ* population was unrealistic. It is plausible that Yang et al. (2006) had a valid argument, particularly given that the last authentic stranding record of a baiji was a pregnant female in 2001, and the last photograph of a baiji was taken in 2002 (Turvey et al., 2007). In contrast, the *ex situ* conservation program set up for the finless porpoise resulted in a very successful outcome with successful breeding and improved conservation outcomes for this species (Wang et al., 2009), as outlined in the following section.

Given the data and the survey findings of Turvey et al. (2007), it is plausible that the baiji had already disappeared, and consequently the arguments put forward by Yang et al. (2006) were valid. However, scientific opinion was divided on how to implement effective conservation

measures to ensure baiji conservation (Reeves and Gales, 2006; Smith et al., 2008; Wang et al., 2006; Yang et al., 2006). Lack of scientific cohesiveness for recovery implementation could have been a factor that contributed to the extinction of the baiji. Turvey et al. (2007) stated that the extinction of the baiji serves as a potent reminder to conservationists that: large, charismatic and nominally protected animals are still at risk of extinction; species cannot be expected to save themselves; and interventions need to be swift and decisive if they are going to succeed. Wang et al. (2006) acknowledged that if *ex situ* measures had been carried out in the 1980's, when baijis were more abundant, then the species recovery programme would have had a greater chance of success.

These lessons with the baiji should provide valuable direction for the MRIDs, so that effective conservation action can be taken to guide remedial action and develop a recovery strategy, as failure to do this for MRID is likely to result in the same outcome as that which befell the baiji. Already the MRIDs are on the same trajectory as the baiji, with a recovery proposal authorised by the Cambodian government in 2009 rejected by the conservation task force appointed in the same year. Similar to the baiji, a series of workshops spanning a decade has been conducted by the conservation task force, with no tangible conservation efforts implemented to save the MRIDs from extirpation.

2.3.2 The Yangtze finless porpoise: an example of a successful *ex situ* recovery program

Both *in situ* and *ex situ* conservation measures were employed for the rescue of the finless porpoise (*N. phocaenoides*) (Mei et al., 2014; Wang et al., 2009). *In situ* conservation measures represent the ultimate objective by allowing porpoises to return to their former

habitats, thus *ex situ* conservation has been regarded as an interim measure to provide sufficient time to restore habitats and the ecosystem function in the Yangtze River (Huang et al., 2017). The feasibility of large-scale *ex situ* developments for recovery have been demonstrated in the Shishou Oxbow Reserve, with 26 finless porpoises successfully captured, maintained and reproducing (Huang et al., 2017; Mei et al., 2014; Reeves and Gales, 2006; Wang 2009; Wang et al., 2006; Yang et al., 2006; Xu et al., 2009; Zhao et al., 2013). However, despite the promise of success, Huang et al. (2017) predicted that extinction of the finless porpoise could still occur, albeit delayed. Their results indicated that the rate of porpoise decline in the Yangtze River may already be too fast and beyond the capacity for reintroduced populations to be able to compensate for these losses, unless the wider Yangtze ecosystem could be drastically improved and restored within a few decades through minimizing anthropogenic threats and reducing the deterioration of water quality (Huang et al., 2017).

2.3.3 Time for action: the precautionary principle

Management of critically endangered species is often delayed due to uncertainties about biological data and anthropogenic effects. For instance, some conservation managers and decision makers undertake precautionary measures, while others delay protective measures until there is strong evidence that an anthropogenic activity is having a serious effect on the species of interest (Berggren and Wang, 2008; Slooten et al., 2000). In such cases, timely reviews of data are required to prevent scientists from literally studying a species to death (Gulland et al., 2020; McGarvey, 2007; Taylor et al., 2019; Torre et al., 2014), as appears to have occurred with the baiji, and is occurring with other critically endangered cetaceans such as the vaquita (Jaramillo-Legorreta et al., 2007; Jaramillo-Legorreta et al., 2019) and the

MRIDs (Beasley et al., 2013; Krützen et al., 2018; Reeves et al., 2009; Ryan et al., 2011). In the case of the vaquita, Jaramillo-Legorreta et al. (2007) stressed that additional data collection would not save the species; instead it would waste valuable funding that was better off used in an intervention and recovery program. This was shown to be true, with the population declining from 150 in 2007 to 19 in 2018 (Jaramillo-Legorreta et al., 2007; Jaramillo-Legorreta et al., 2019). The costs associated with collecting more data for critically endangered populations are two-fold. Firstly, the cost of data collection and holding scientific meetings is high and significantly reduces funds needed to implement effective recovery initiatives (Jaramillo-Legorreta et al., 2019). Secondly, the time required to collect and analyse such data comes at the expense of the species that is in peril.

Making decisions on the basis of sparse data is common in conservation (Falcy, 2016; Slooten et al., 2000). For example, action has been required to mitigate against worldwide amphibian declines, prior to the full "story" being understood (Ludwig et al., 2001). Unfortunately, the effect of limited data can also have grave consequences, despite the best intentions. For example, Taylor and Gerrodette, (1993) found that implementing intervention strategies based on accepting an incorrect or incomplete diagnosis for small critically endangered populations can be acute, as these populations have limited margin for recovery from incorrect management decisions. Conservation managers should be cognisant of the effect of false negative results, which potentially could result in the extinction/extirpation of a species (Taylor and Gerrodette, 1993; Falcy, 2016). Thus, when it comes to the conservation of a declining critically endangered species/population, it is best practice to use the precautionary principle to give the imperilled species the best chance of survival.

2.4 Investigative framework:

This final section of the literature review examines the framework that can be used to investigate mortality and extirpation risk in the MRIDs. In particular, conservation medicine was chosen as the overarching framework for the studies reported in this thesis.

2.4.1 Conservation medicine

Conservation medicine is a reasonably new scientific discipline that encompasses disease ecology and its application to wildlife species conservation, particularly focusing on biodiversity loss as a consequence of imbalances in the ecosystem, and human and animal health (Aguirre et al., 2012; Jakob-Hoff and Warren, 2012; Mangini et al., 2012; Norris, 2001). It is designed to improve ecological health, and thus worldwide health for all living things (Dierauf et al., 2001). Hudson (2003) described conservation medicine as the consequence of the human footprint on the ecological balance of disease dynamics. As such, conservation medicine is essentially a response to the emergence of new diseases and physiological threats to animals and people, caused by industry, agriculture and development, which are contributing to species decline (Soulé, 2002).

The term conservation medicine was first used by Koch (1996); however a uniformly accepted definition of conservation medicine is still evolving (Aguirre et al., 2012). Vitali et al. (2011) identified three core principles of the discipline. Firstly, it encompasses the study of ecological health. For example, conservation medicine aims to address health problems derived from environmental change and anthropogenic insults to the environment (Daszak et al., 2006). Secondly, it facilitates the interpretation of information pertaining to both health and disease. This is achieved by utilising existing knowledge frameworks in wildlife health,

public health, epidemiology, ecology, conservation biology, and veterinary science (Aguirre et al., 2012; Aguirre et al., 2020; Daszak et al., 2006,). Finally, it uses a multidisciplinary approach, including the fields of climatology, anthropology, economics and political science (Alders, 2009), as well as those listed previously, in order to be successful. Through this multidisciplinary approach, conservation medicine is able to apply multiple tools to investigate and assess the health status of a population (Tabor, 2002; Mangini et al., 2012). Thus, by working on the bigger picture, conservation medicine provides context for these more specialised disciplines to interact effectively (Daszak et al., 2006).

Conservation medicine closely allies with conservation biology, as health and disease are fundamentally related to the integrity of the ecosystem (Alders, 2009). Conservation biology addresses the ecology of species, communities, and ecosystems that are affected, either directly or indirectly, by anthropogenic activities, with an overarching goal of providing principles and tools for preserving biological diversity (Soulé, 1985), whilst conservation medicine uses the application of medicine to augment the conservation of wildlife and their ecosystems (Deem et al., 2000).

2.4.1.1 River dolphins: sentinels of ecosystem health

One premise of conservation medicine is that anthropogenic changes to ecosystems are one of the primary factors leading to disease outbreaks in both wild and domesticated animal populations (Mangini et al., 2012). As such, disease at the population level is a sign of an imbalance in, or insult to, the ecosystem (Meffe, 1999). Thus, conservation medicine represents a shift from viewing diseases in wildlife or humans simply as an individual response to pathogens, to taking a holistic view of understanding them as broader complex

processes, affected by ecological systems (Meffe, 1999). In this context, wildlife, such as birds (Smits and Fernie, 2012), dolphins (Hart et al., 2020) and frogs (Maselli et al., 2010), can serve as important sentinels of environmental health status and pollution (Cooper, 2002). River dolphins are particularly good sentinel species for monitoring the health of large tropical rivers that are impacted by human generated stressors (Gomez-Salazar et al., 2012), because they have a long life span, are long-term residents, feed at a high trophic level, and have unique fat stores that can serve as depots for anthropogenic toxins (Bossart, 2011; de Guise et al., 2003; Moeller, 2003; Reif, 2011; Ross, 2000; Ross and Birnbam, 2003; Wells et al., 2004). Thus, dolphins can be used to monitor the long-term effects of pollution on their aquatic environment (Mössner and Ballschmiter, 1997), to evaluate the health of these aquatic ecosystems (Bossart, 2011) and be global pollution indicators (Mössner and Ballschmiter, 1997).

2.4.1.2 Surveillance and monitoring of wildlife diseases

The need for surveillance programs for diseases in wildlife populations is becoming more evident (Mörner et al., 2002), particularly with diseases such as covid (Aguirre et al., 2020; Gaynor et al., 2020; Lewis, 2021), SARS (Feng et al., 2009; Merianos et al., 2008; Page and Frieman, 2001), swine influenza (Ding et al., 2021; Dubey et al., 2009; Li and Robertson, 2021; Michaelis et al., 2009; Schnitzler and Schnitzler, 2009; WHO, 2011), avian influenza (Capua and Alexander, 2007; de Jong, 2007; Khan et al., 2018; Lai et al., 2016; Swayne, 2008), and henipaviruses including Hendra and Nipah (Barclay and Paton, 2000; Breed et al., 2006; Daniels et al., 2007; Eaton, 2001; Eaton et al., 2005; Field et al., 2007; OIE, 2010; Plowright et al., 2011; Singh et al., 2019; Wong et al., 2007; Yuen et al., 2021), spreading between wildlife and domesticated animals and vice versa and also to people. Mörner et al.

(2002) stated that reports of mass mortalities in a wildlife population, such as the MRIDs, may represent the initial alert to the likelihood of a new disease agent, and may be an important indicator of an ecological disturbance, introduction of a new animal species, climatic or habitat change, or local pollution, and as such early investigation and intervention of these events is essential to determining the cause and significance of such outbreaks. Surveillance and monitoring programs are the first steps towards providing an appropriate level of understanding of the health status of any population, as the presence or absence of an infection/disease cannot be declared unless sampling and statistical analyses are undertaken (Mörner et al., 2002).

2.4.1.3 Monitoring mortality events in wild populations

Mortality events involving wildlife often occur unpredictably and opportunities to investigate these events may be short-lived (Mörner et al., 2002), such as with stranded or fisheries entangled marine mammals (Bossart et al., 2007; Braulik et al., 2010; Diaz-Delgado et al., 2018; Domiciano et al., 2016; Jefferson et al., 2006). Samples collected opportunistically from such events may provide some insight into the occurrence of important disease processes in these wild populations (Mörner et al., 2002), as has been shown with pox virus in cetaceans (Cocumelli et al., 2017). Characterisation of the environmental conditions associated with disease and disease outbreaks is an important part of every investigation and is relevant to understanding the epidemiology of diseases in wildlife (Mörner et al., 2002), as has been demonstrated in studies on white nose disease in bats (Flory et al., 2012) and chytridiomycosis in frogs (Scheele et al., 2019).

The prompt detection and effective management of disease in wildlife relies greatly on field diagnosis, with pathological examination (gross necropsy with supporting laboratory investigations) being the cornerstone of the diagnosis and investigation of wild animal diseases and unusual mortality events (Cooper, 2002; Terio et al., 2018; Warns-Petit et al., 2010; Pierre and Degiorgis, 2013). Gross post-mortem and laboratory investigations of specimens collected from wildlife provide an opportunity for the early diagnosis and detection of endemic and new or emerging conditions (Cooper, 2002; Duncan 2001; Akdesir et al., 2018), provided they are carried out systematically and in accordance with strict protocols to ensure sample collection is standardised (Cooper, 2002; Duncan 2001; Rowles et al., 2001). For instance, laboratory tests are useful for the diagnosis of infectious and non-infectious diseases, particularly when the population is suffering high mortality (Cooper, 2002), as has been shown in studies by Akdesir et al. (2018) in mustelids, and Esquible et al. (2019) in Steller Sea Lions (*Eumetopias jubatus*). In addition, such investigations can also detect: subclinical disease; underlying pathological lesions which may be affecting survival; reproductive success; assist in developing reference ranges; and permit the establishment of reference collections (banks) of tissues (Cooper, 2002, Wells et al., 2004).

2.4.1.4 River dolphins in the context of conservation medicine and disease surveillance

Conservation medicine is gaining acceptance as a framework that encompasses the complexity of disease ecology and applies it to the conservation of wildlife (Jakob-Hoff and Warren, 2012). In this context, threats to the MRIDs and their conservation need to be better understood. Such threats may include: anthropogenic activity; chemical pollution; presence of domesticated animals such as buffalo, and their pathogenic agents (Mangini et al., 2012); and global changes such as climate change (Price et al., 2019). Veterinarians dealing with

dolphin health problems *in situ* should integrate a wide range of tools and knowledge from different fields into their wildlife health surveillance and research, in order to understand the impact of anthropogenic influences and their correlation with health risks and disease prevalence (Mangini et al., 2012; Pierre and Degiorgis, 2013). For instance, understanding the MRIDs' ecology and how they live in their natural habitat can improve understanding of the epidemiology of disease in the population (Mangini et al., 2012, Zhao et al., 2013). As such, strategies to deal with mortality issues require knowledgeable practitioners with well-developed skills and tools for data collection from multiple disciplines (Mangini et al., 2012; Reading et al., 2013) including pathological, histological, microbiological, epidemiological, toxicological and genetic investigations.

2.4.1.5 Dolphin health assessment

Health assessments in dolphins are complex, in that in addition to obvious threats such as accidents/bycatch, disease, predation, and infanticide, there are insidious threats, such as contaminants and genetic threats, that are not only manifested as obvious congenital defects (Lacy, 1997) but may also result in reduced reproductive success.

Dolphins live in a hostile, aquatic environment, and need the fullest complement of their special sensory and behavioural systems to ensure their reproductive success and survival (Colborn and Smolen, 2003). Yet the protected status of cetaceans renders the direct health assessment of wild populations difficult (Yordy et al., 2010a). In addition, the logistics of working with live cetaceans is often impossible or ethically challenging, so alternatives are needed to determine the anthropogenic hazards posed to cetaceans by the growing list of xenobiotics that can affect their development and function (Colborn and Smolen, 2003).

Thus, health assessments need to incorporate a toxicological component, and should include the impact on an individual's immune, endocrine, nervous and reproductive systems, and the developing brain of young (Colborn and Smolen, 2003).

Small populations may also be vulnerable to demographic and genetic stochasticity, which can accelerate the process of extinction or regional extirpation, even when effective conservation measures are in-place to protect the dolphins and their habitat (Rosel and Reeves, 2000). Thus, investigations of extinction/extirpation risk, or recovery plans for threatened taxa need to evaluate genetic factors (Frankham, 2005; Frankham et al., 2014), and awareness of the genetic and demographic consequences of small population size should be integral to conservation planning (Frankham et al., 2014; Rosel and Reeves, 2000), particularly in critically endangered river dolphin populations, such as the MRIDs.

2.4.2 Conservation genetics

Studies of the genetics of critically endangered populations can play a vital role in conservation planning. Such studies provide insights into inter- and intra-population relationships, movement of individuals between populations, population demography, interactions among individuals, and the nature of breeding systems (Rosel and Reeves, 2000), particularly as conservation genetics takes into account the historical and current structure of a population, both in a demographic and phylogenetic sense (Pertoldi et al., 2007).

Conservation genetics facilitates the understanding of genetic erosion in the extinction/extirpation risk of a population (DeSalle and Amato, 2004; DeYoung and Honeycutt, 2005; Frankham, 1995a; Frankham et al., 2002; Hedrick, 2001; Hendrick and

Kalinowski, 2000; Kohn et al., 2006; Lande, 1988; Lynch, 1996; Pertoldi et al., 2007; Spielman et al. 2004; Weeks et al., 2016). Specifically, neutral genetic markers, defined as gene sequences where variants confer no fitness advantage (Stouthamer and Nunney, 2014), are widely used to assess inbreeding levels, genetic variation, population structure and phylogenetic conservation units (Pertoldi et al., 2007). The two most commonly used markers are mitochondrial DNA and microsatellite sequences that have high mutation rates (Pertoldi et al., 2007).

Recently, population genomics has gained momentum and is being applied in the conservation and management of wildlife species (Funk et al., 2016; Gallego-García et al., 2018; Hohenlohe et al., 2021; Kohn et al., 2006; Shafer et al., 2015), and examination of adaptive markers, including genes of the major histocompatibility complex (MHC), may be better markers for genetic variability (Manlik et al., 2019) than neutral markers. Genomics can be used to determine effective population size, inbreeding, demographic history, and population structure, and can be used to identify particular genetic loci and variants responsible for inbreeding depression or adaptation (Hohenlohe et al., 2021; McMahon et al., 2014). The genomic approach offers a dramatic increase in the number of variable genetic markers used (generally single nucleotide polymorphisms - SNPs), as such it can bypass the laborious process of marker characterisation, primer development, and genotyping required for microsatellites and can be compared and combined across laboratories, which is not always possible with microsatellite loci (Shafer et al., 2015). As such, both genomics and MHC studies should be considered in any future genetics projects for the MRIDs.

2.5 Conclusions

Investigating the MRID population decline requires studies covering a broad range of disciplines. The results of such studies form the basis of the chapters included in this thesis. The overall conservation and management of the MRID population depends, in part, on the ability to integrate knowledge from multiple disciplines, including conservation medicine, disease surveillance, epidemiology, and conservation genetics, in order to identify potential risks for mortality and population decline. The studies incorporated in this thesis draw on extant literature as provided in this chapter, together with retrospective mortality data, necropsy case studies, epidemiology, eco-toxicology, and conservation genetics, to define the threats to the MRID population. The results from these studies help identify the key drivers influencing the mortality of the MRID population so that better conservation management decisions can be made. The results of the health assessment can then be used to direct effective conservation strategies to mitigate further population decline. The concepts and theories described in this literature review provide the background and context for the multidisciplinary approach used in this study of MRID.

CHAPTER 3

An epidemiological investigation of mortality in a population of Mekong River Irrawaddy dolphins over a 10-year period in Cambodia.

3.1 Introduction

Over the past decade there has been considerable scientific debate over the reasons for the population decline in MRIDs and the associated causes of mortality (CSG 2012a; 2012b; Gulland, 2009; 2014; IUCN-CSG, 2012; Krützen et al., 2018; Reeves et al., 2009; Schnitzler et al., 2021; Smith, 2017; Thomas and Gulland, 2017; Wells, 2014; WWF et al., 2012; 2014). The mortality trend in this population is unsustainable and the population faces extirpation if the mortality rate cannot be mitigated against (Beasley, 2007; Caballero et al., 2019; Dove et al., 2007; Ryan et al., 2011; Vibol et al., 2009; Krützen et al., 2018).

As outlined in the previous chapter there are multiple potential explanations for the decline in the MRID; however, researchers have failed to agree on the key risk factors for this population decline. Beasley (2007) reviewed fisheries-related mortality in adult *O. brevirostris* in the Mekong River, and found that accidental entanglement in gillnets was the major cause of mortality. Beasley (2007) also found that, in contrast to the observations in adults, there was a lack of evidence of entanglement in dead calves, even though calves accounted for 43% (n=54) of the reported mortalities in the MRID population. In contrast, the IUCN conservation task force considered that interaction with fisheries activities was responsible for mortalities in all dolphin age classes (Reeves et al., 2009; Smith, 2017;

Thomas and Gulland, 2017). These conflicting conclusions highlighted the need for research to identify the primary causes of mortality in the population, especially in calves.

In this chapter historical mortality data of 102 dolphins for the decade 2001 to 2010 are analysed to compare mortality in different age groups of MRIDs (calves, juveniles and adults) so that effective conservation management strategies can be developed and implemented to reduce or reverse the population decline.

3.2 Materials and methods

Dolphin mortality data, covering the period January 2001-March 2010, were collected by two teams. The first included Isabel Beasley and Martin Gilbert, in association with the Wildlife Conservation Society (WCS) (from 2001-Oct 2006), and the second I personally led from November 2006 to March 2010 in collaboration with the Fisheries Administration of Cambodia (MAFF), WCS and the WWF (Beasley, 2007; Beasley and Gilbert, 2005; Beasley et al., 2009; Dove, 2008). When permitted by the Cambodian Government, comprehensive data collection on each carcass was conducted including morphometrics, photographs and necropsies. These data were compiled into a database and subsequently analysed. In total, there were 102 confirmed dolphin mortalities during this period. A summary of the 102 carcasses included in this study is outlined in Appendix 1: Table 1. Each dolphin was given a unique field code (OBRE from 2002 to 2006 or CID from 2006 to 2010). The 102 dolphins used in this study have been given an identification number (ID #) for ease of identification throughout this thesis.

Of the 102 dolphin carcasses, 69 were photographed to better document the mortality event. Additionally, necropsy examinations were conducted on 41 carcasses by trained veterinarians, with the remaining 61 not having a professional necropsy exam for several reasons, including lack of a permanent in-country veterinarian prior to November 2006, authorisation denied by the Cambodian government, or the carcass was too decomposed. Gilbert and Beasley performed necropsies on 14 dolphins from 2004-October 2006 and I performed 27 necropsies on dolphins from November 2006 to March 2010.

The age and necropsy distributions over time for the entire dataset are shown in Figure 3-1.

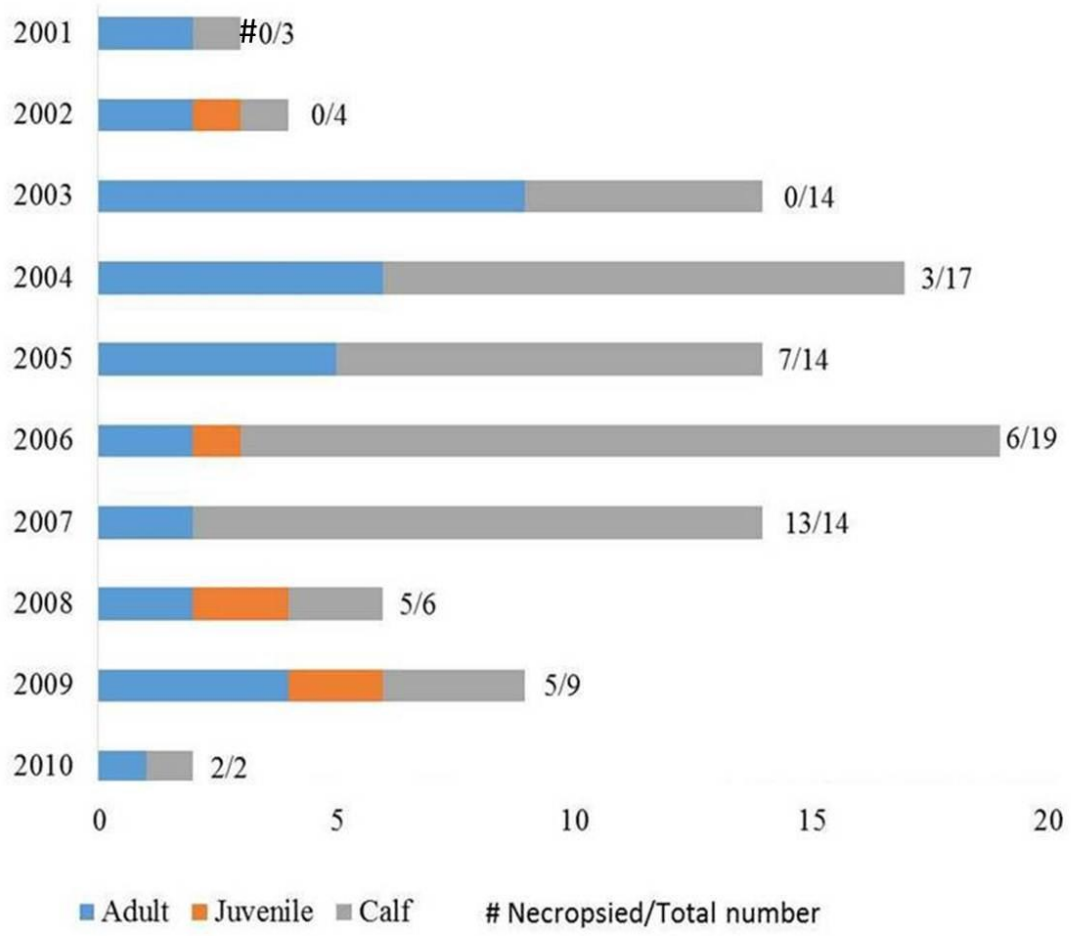


Figure 3-1: Summary of the mortality data* from 2001-March 2010 (n=102 dolphins).
*Data Source:
2001-2005: Beasley, 2007; Beasley and Gilbert, 2005; Beasley et al., 2009.
2006: Beasley, 2007; Beasley et al., 2009; Dove, 2008.
2007-2008: Dove, 2008; 2009.
2009: Dove, 2009

Based on external examination of the carcass the research team assigned a decomposition carcass score (CS), ranging from 1 (live) to 5 (severe decomposition), for every carcass using a score adapted from Rowles et al. (2001) (Table 3-1). A CS1 was allocated to those dolphins that were initially alive but died within 24 hours. These dolphins were subsequently given a score of SC2 on their death.

Table 3-1: Classification of decomposition carcass score adapted from Rowles et al. (2001)

Carcass score (CS)	**Presentation**	**Gross appearance**	
		External	Internal
1	Live	Animal still alive when found.	
2	Freshly dead	No bloating. Minimal drying of the epidermis, eyes and mucous membranes.	Muscles firm. Blubber firm and white or yellow in colour. Internal organs intact; physical integrity of liver retained
3	Moderate decomposition	Slight bloating with tongue protruding, along with penis protruding in males. Epidermis shows evidence of sloughing and cracking. Eyes sunken.	Blubber may be blood-tinged. Muscles soft. All internal organs, including liver, maintain gross integrity but are soft and friable.
4	Advanced decomposition	Bloated. Missing patches of epidermis. Pooled oil within body cavities from blubber.	Blubber with gas pockets. Internal organs show lack of integrity and are extremely friable.
5	Severe decomposition	Mummified or skeleton.	

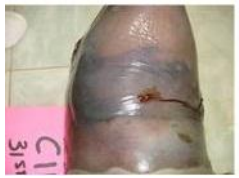
The lesions/conditions detected in the carcasses were classified into five broad categories: neck lesions; disease (other than neck lesions); fisheries related activities; neck lesions and

fisheries related activities; or “could not be determined” (CNBD) (Table 3-2). Both neck lesions and cases of disease without neck lesions were classed together broadly as ‘disease’ in this study and thesis.

Neck lesions were variable, differing in size, extent and colour as shown in the images of 12 cases in Figure 3-2.

Figure 3-2: Examples of neck lesions seen in a selection of carcasses.
(Photographs provided by MAFF, WCS-Cambodia and WWF-Cambodia).

Table 3-2: Classification of five broad carcass conditions categorised in this study

Classification	Definition	Gross appearance	Examples
Neck lesions	Presence of discolouration on the ventral neck area.	Red to blue/black discolouration of the dermis or epidermis. Gas gangrenous changes and darkened colouration on cut sections of the neck musculature, noticeably different to the rest of the musculature of the body.	 
Disease	Gross evidence of a disease process/lesion (other than a neck lesion) detected with characteristics of a disease process, with or without microbiological/histological confirmation.	Any gross pathological condition that was visible on external or internal examination.	
Fisheries	Evidence of fisheries interaction or entanglement.	Fishing net entangled on the dolphin carcass, lacerations consistent with fishing nets/gear/lines.	
Neck lesions & fisheries	Presence of discolouration on the ventral neck area in addition to evidence of fisheries interaction or entanglement.	Combination of classifications as described above.	
Could not be determined (CNBD)	No obvious external or internal abnormalities detected that could be attributed to the cause of death	Lack of all the above conditions.	

3.2.1 Exclusion parameters

The data were standardised by omitting carcasses in CS 4 or 5 because with greater decomposition it was difficult to draw inferences about potential underlying pathology and causes of mortality. Only dolphins which were scored as CS2 (n=23) or CS3 (n=23) were included in the analysis (n=46). Five of these 46 CS2 or CS3 dolphins were subsequently categorised as CNBD and were excluded, resulting in 41 dolphins included in the analysis.

3.2.2 Necropsied dolphins in carcass score S2 or 3

Of the 41 dolphins that were necropsied, 32 were CS2 or 3. Four of these 32 dolphins were excluded where the cause of death was recorded as CNBD, resulting in 28 dolphins included in this component of the analysis.

3.2.3 Independent replication using only photographic evidence

Given the potential controversy and variability in the aetiology of dolphin mortality, William McLellan of the University of North Carolina, Wilmington was commissioned by the IUCN task force and WWF to further validate the study observations in an independent replication (McLellan, 2010 pers. comm.). McLellan was given all photographs from the database of 69 dolphins and he then scored the quality of the photographic images (score 1 - highest quality to 3 – lowest quality), the individual animal's CS, and the major cause of death based on these photographs.

In both studies each of the 69 dolphins were allocated to one of three categories: Firstly, those with evidence of fisheries interaction (FI) (including: entanglement, fishing-nets on the body, or obvious net marks that clearly indicated interaction with fisheries activities); secondly, those confirmed as not having any evidence of fisheries interaction, categorised as non-fisheries interaction (NFI); and finally, those that were either too decomposed to determine or a judgement could not be made. This latter group were recorded as could not be determined for fisheries interaction (CNBD-FI). In this study, dolphins reported by locals as likely caught in fishing nets with no physical evidence on examination or photographs of fisheries interaction were also recorded as CNBD-FI.

McLellan's results from examination of the photographs were then compared to the mortality database for the same dolphins in CS2 or 3 (n=46), and to those that were also necropsied (n=30) as part of the current study. Both sets of data were standardised by omitting the dolphins that were categorized as CNBD-FI.

3.2.4 Statistical and epidemiological analyses

Data were analysed to determine the association between age and gender on the findings on necropsy using 2x2 contingency tables. Prevalence and their associated 95% confidence intervals (95%CI) were calculated for the various age categories and necropsy findings. Both the Pearson's Chi-square test for independence and odds ratios (OR) and their 95%CI were used to identify statistical differences in categorical data (Kahn and Sempos, 1989). The Fisher's exact test was used on smaller data sets to determine significant differences between groups using the Real Statistics Resource

Pack (Release 5.4) for Excel (2010) (Zaiontz, 2018). A McNemar-Bowker Test was used for correlated proportions in paired samples using a 3x3 contingency table to identify statistical differences using Statistix version 10.0 (Analytical Software, Florida).

3.2.4.1 Gillnet associated mortality

Incidence risk for mortality from any cause was calculated for adults and for calves over the three year period when gillnets were used (2003-2005) and compared to the incidence risk for the three year period when the use of gillnets was restricted (2006-2008). The adult population at risk at the start of the period (2003) was estimated by adding the number of deceased adult dolphins reported each year to the number estimated to be present at the end of 2008 (83). This resulted in an estimated at-risk population of 109 adults in 2003 and 89 dolphins in 2006. Relative Risk (RR), Attributable Risk (AR) and Attributable Fraction (AF) and their associated 95%CI were calculated. The calf population at risk was also calculated for the same three year periods using two methods. The maximum population size was estimated by assuming 16 calves were born each year during the period. This value was the largest number of dead calves detected in one of the study years (2006). The annual average number of dead calves detected during the period was also calculated (n=12) and this value was used to estimate the most likely (average) population size for calves.

3.3 Results

3.3.1 All data

From 2001 until March 2010, there were 102 documented and confirmed MRID mortalities in Cambodia where the carcasses were retrieved or photographed. Of the 102 dolphins, more calves (59.8%; 95%CI 49.6, 69.4) were presented than adults (34.3%; 95%CI, 25.2, 44.4) and juveniles (5.9%; 95%CI 2.2, 12.4).

3.3.2 Dolphins in carcass score 2 or 3

The results are summarised in Table 3-3. Of the 41 dolphins in CS2 or 3 analysed in this study, there were more calves (58.5%; 95%CI 42.1, 73.7) than adults (34.2%; 95%CI, 20.1, 50.6) and juveniles (7.3%; 95%CI 1.5, 19.9). Overall there was a significant difference between the three age categories for the number of deaths from the four mortality categories ($\chi 2$ (d.f. 2, 3, n=41) = 17.4, p=0.008).

In this study, the odds of dead calves having a neck lesion was 15 times higher than that for dead adults (OR 14.7; 95%CI 2.44, 88.13; Fisher's exact test p=0.001). Overall there was a significant difference in the number of dolphins in the three age groups with neck lesions ($\chi 2$ (d.f. 1, 2, n=41) =10.8; p=0.004). However, there was no significant difference between the presence of lesions in juveniles and adults (OR 2.67; 95%CI 0.19, 36.76, p=0.57). There was also no significant difference found in the proportion of dead male dolphins with neck lesions (43%) than in dead females (56%) (OR 1.2: 95%CI 0.32, 4.4; p=0.79).

In this study, dead adults were 17 times more likely to have evidence of fisheries interaction than dead calves (OR 17.25; 95%CI 1.79, 166.1, Fisher's exact test

p=0.006), and overall the proportion of fisheries related deaths in the three age groups were significantly different ($\chi 2$ (d.f. 1, 2, n=41) =10.05, p=0.006).

3.3.3 Necropsy data for dolphins in carcass score 2 or 3

The results are summarised in Table 3-4. Of the 28 dolphins in CS2 or 3 necropsied there were more calves (68%; 95%CI 47.6, 84.1) than adults (25%; 95%CI, 10.7, 44.9) or juveniles (7%; 95%CI 0.8, 22.1). None of the 28 dolphins were classed as only having fisheries interaction, and so this category was omitted from the analysis. Overall there was a significant difference between the three age categories for the number of deaths from the three remaining mortality categories (Table 3-4; $\chi 2$ (d.f. 2, 2, n=28) =19.84, p=0.0005). Furthermore, there was a significant difference in the number of dolphins in the three age groups with neck lesions ($\chi 2$ (d.f. 1, 2, n=28) =7.84; p=0.019)

Table 3-3: Comparison of the cause of death in 41 CS2 or 3 Mekong River Irrawaddy dolphins categorised into different age groups.

Potential cause of mortality		n	Total proportional mortality ratio % (95%CI)	Age group	n	Age group-specific prevalence in age class % (95%CI)	Odds ratio (95%CI)	P-value*
Disease	Neck lesions (NL)	30	73.2 (57.1, 85.8)	Adults	6	42.8 (17.7,71.1)	1	0.0045
				Juveniles	2	66.7 (9.4,99.2)	2.67 (0.19, 36.76)	
				Calves	22	91.7 (73.0, 99.0)	14.67 (2.44, 88.13)	
	Disease & No NL	3	7.3 (1.5, 19.9)	Adults	2	14.3 (1.8, 42.8)	1	0.0526
				Juveniles	1	33.3 (0.8, 90.6)	3 (0.18, 50.79)	
				Calves	0	0 (0,14.2)	-	
Fisheries	Fisheries only	7	17.1 (7.2, 32.1)	Adults	6	42.9 (17.7, 71.1)	17.25 (1.79, 166.10)	0.0067
				Juveniles	0	0 (0, 70.8)	-	
				Calves	1	4.2 (0.1, 21.1)	1	
	Fisheries & NL	1	2.4 (0.1, 12.9)	Adults	0	0 (0, 23.2)		0.70
				Juveniles	0	0 (0, 70.8)		
				Calves	1	4.2 (0.1, 21.1)		

* Results from a Pearson's χ^2 of the difference between age groups for each category. Overall there was a significant difference between groups χ^2 (df 2, 3, n=41) = 17.4, p=0.008.

Table 3-4: Comparison of the cause of death in 28 necropsied CS2 or 3 Mekong River Irrawaddy dolphins categorised into different age groups

Potential cause of mortality		**n**	**Total proportional mortality ratio %(95%CI)**	**Age group**	**n**	**Age-specific prevalence % (95%CI)**	**P-value***
Disease	Neck lesions (NL)	25	89.3 (71.8, 97.7)	Adults	5	71.4 (29, 96.3)	0.019
				Juveniles	1	50 (1.3,98.7)	
				Calves	19	100 (82.4, 100)	
	Disease & no NL	2	7.14 (0.9,23.5)	Adults	2	28.57 (3.7, 71)	0.039
				Juveniles	0	0 (0, 84.2)	
				Calves	0	0 (0, 17.6)	
Fisheries	Fisheries only	0	0 (0, 12.3)	Adults	0	0 (0, 41)	
				Juveniles	0	0 (0, 84.2)	
				Calves	0	0 (0, 17.6)	
	Fisheries & NL	1	3.57 (0.1, 18.3)	Adults	0	0 (0, 41)	0.001
				Juveniles	1	50 (1.3,98.7)	
				Calves	0	0 (0, 17.6)	

* Results from a Pearson's χ^2 test of the difference between age groups for each disease category. Overall, after excluding fisheries only, there was a significant difference between groups χ^2 (df 2, 2, n=28) =19.84, p=0.0005.

3.3.4 Comparison of necropsy results with independent assessment of photographic evidence

The images of 69 dolphins were scored by McLellan, with 61 (89%) classified as in score 1, 5 (7%) in score 2, and 3 (4%) in score 3. Of these 69 photographed animals 20 were adults, three were juveniles, and 46 were calves. Only adults were identified as being entangled in gillnets.

This study found there was no significant difference between examining photographic evidence with necropsy data for evidence of fisheries interaction for the 30 dolphins both procedures were applied to (p=0.99, Table 3-5), or in evaluating the carcass condition (CS2 or 3) (χ^2 (d.f. 1, 1 n=30) = 0.135, p=0.7125).

Table 3-5: Comparison of McLellan's photographic study and the current necropsy data of 30 Mekong River Irrawaddy dolphins

		Photographic study			
		Fisheries interaction	**No fisheries interaction**	**CNBD-FI**	**Total**
Necropsy study	**Fisheries interaction**	4	0	10	14
	No fisheries interaction	0	1	0	1
	CNBD-FI	11	0	4	15
	Total	15	1	14	30

CNBD-FI: Could not be determined for fisheries interaction

As there was no difference found between the two methodologies, the findings from all the data, the dolphins in CS2 or 3 and the necropsied dolphins in CS2 or 3 were compared. The results of the comparative findings are summarised in Table 3-6. No differences were found between the two methodologies for any of the observed conditions (all $p>0.05$ as evident by the means and their 95%CI).

3.3.5 Gillnet associated mortality

The incidence of mortality (from any cause) in adults for the three year period when gillnets were used was 18.3% (95%CI 11.6, 26.9), and this reduced to 6.7% (95%CI 2.5, 14.1) when gillnet restrictions were in place. The RR of dying during the three years with gillnets was 2.7 times (95%CI 1.1, 6.5) that during the three year period when gillnet use was restricted ($p=0.016$). Adult mortality could be reduced by 11.6% (95%CI 7.5, 16.9) (AR) if adults were not exposed to gillnets, and 63.3% (95%CI 55.9, 69.7) of adult mortality was attributed to the exposure of gillnets (AF).

In contrast to adults, there was no significant difference in the incidence of mortality (from any cause) for calves between the three year period when gillnets were in place and in the three year period when they were restricted (72.2%, 95%CI 54.8, 85.8 for 2003-2005 and 83.3%, 95%CI 67.2, 93.6 for 2006-2008 assuming 12 calves born per annum ($p=0.39$); and 54.2%, 95%CI 39.2, 68.6 for 2003-2005 and 62.5%, 95%CI 47.4, 76.0 for 2006-2008 assuming 16 calves born per annum ($p=0.53$)).

Table 3-6: Summary data comparing the findings on photographic analysis (McLellan 2010) with the gross examination results in 69 Mekong River Irrawaddy dolphins

	All mortality data				CNBD-FI data excluded			
	This study		McLellan		This study		McLellan	
	n	% (95%CI)	n	% (95%CI)	n	% (95%CI)	n	% (95%CI)
All data								
No fisheries interaction	17	24.6 (15.1, 36.5)	24	34.8 (23.7, 47.2)	17	63 (42.4, 80.6)	24	72.7 (54.5, 86.7)
Fisheries interaction	10	14.4 (7.2, 25)	9	13.0 (6.1, 23.3)	10	37 (19.4, 57.6)	9	27.3 (13.3, 45.5)
CNBD-FI	42	61 (48.4, 72.4)	36	52.2 (39.8, 64.4)				
Total	**69**		**69**		**27**		**33**	
CS 2/3 only								
No fisheries interaction	16	34.8 (21.4, 50.2)	22	47.8 (32.9, 63.1)	16	72.7 (49.8, 89.3)	22	81.5 (61.9, 93.7)
Fisheries interaction	6	13.0 (4.5, 26.3)	5	10.9 (3.6, 23.6)	6	27.3 (10.7, 50.2)	5	18.5 (6.3, 38.1)
CNBD-FI	24	52.2 (36.9, 67.1)	19	41.3 (27, 56.8)				
Total	**46**		**46**		**22**		**27**	
Necropsied dolphins CS 2/3								
No fisheries interaction	14	46.7 (28.3, 65.7)	15	50 (31.3, 68.7)	14	93.3 (68.1, 99.8)	15	93.8 (69.8, 99.8)
Fisheries interaction	1	3.3 (0.1, 17.2)	1	3.3 (0.1, 17.2)	1	6.7 (0.2, 31.9)	1	6.3 (0.2, 30.2)
CNBD-FI	15	50 (31.3, 68.7)	14	46.7 (28.3, 65.7)				
Total	**30**		**30**		**15**		**16**	

3.4 Discussion

This study found evidence that the category of lesions present on necropsy differed between calves, juveniles, and adult dolphins. Dead adults were more likely to show evidence of interaction with fisheries activities, whilst neck lesions were more commonly associated with the dead calves. In contrast to age, the presence of lesions/interactions was similar between the sexes.

3.4.1 Key findings

This is the largest study conducted to date that has examined mortality data of MRID and resulted in several important findings. This is also the first comparison of lesions/conditions in different age classes of deceased MRID, with significantly more adults found to have evidence of interaction with fisheries activities than calves or juveniles, and significantly more calves having neck lesions than adults and juveniles.

The current study also found that, in order to determine evidence of fisheries interaction, there was no difference between using photographs to assess dolphin carcasses and examining animals by performing a necropsy. This is a very important finding as it facilitates future studies to use photography to evaluate the extent of interaction with fisheries activities in the population. However, more dead dolphins need to be compared by both methods and full necropsies conducted, including virological testing, to fully compare these two methods for pathological assessment. The results in this study demonstrate the benefit of combining methodologies (necropsies, photographs, and external observations) for the overall assessment of retrospective mortality data for the MRIDs, and potentially other marine mammals.

3.4.1.1 Fisheries interaction

This study found that dead dolphins that had evidence of fisheries interaction were more likely to be adults than calves. These findings are consistent with the research findings of Beasley (2007); however did not agree with the conclusions made by the IUCN conservation task force which reported that gillnet entanglement was the principal cause of mortality in the MRID population (Reeves et al., 2009; WWF et al., 2012). Human-induced mortality from fisheries has been identified as the predominant threat worldwide to a range of dolphin species (Burkhart and Slooten, 2003; Choudhury et al., 2019; Cox et al., 1998; Iriarte, 2013; Kiszka et al., 2008; Loch et al., 2009; Mansur et al., 2008; Moore and Read, 2008; Slooten and Davies, 2012; Slooten et al., 2000; Wade and Slooten, 2020), however the results in this study demonstrate strong evidence for different causes of mortality in different age classes for MRIDs.

3.4.1.2 Neck lesions and implications for future research

3.4.1.2.1 Neck lesions in calves

There has been little consensus as to the potential aetiology of the neck lesions in MRIDs or their role in dolphin mortality (CSG, 2012a; Dove, 2009; Gulland, 2009; 2014; McLellan, 2010 pers. comm.; WWF et al., 2012). However, in the current study neck lesions were predominant (91.7%) among the dead calves.

The evidence presented here supports the conclusions of McLellan (2010 pers. comm.) and the IUCN-conservation task force (CSG, 2012a) that the neck lesions are unusual and

uncharacteristic in dolphins, and that a very high proportion of the necropsied calves have what McLellan called ‘stereotypical neck lesions’. The study’s observations specifically corroborate the photographic findings of McLellan (2010 pers. comm.) who reported that the vast majority of dead neonatal calves examined had a ventral neck lesion not associated with fisheries activities.

This study also found evidence that was inconsistent with the hypotheses of Reeves et al. (2009) and Gulland (2009) who argued that fishing “gear” was attributable for most of the recorded calf mortalities, claiming that the linear traumatic lesions associated with bruising of the neck was associated with injuries arising from this “gear”. Furthermore, the current study identified the presence of anatomical neck folds uniquely found in the calves of this species, and not previously documented in the literature (Appendix 2), which may have been mistaken for the linear lesions described by Gulland (2009). However, the description of bruising associated with the neck lesions, as suggested by Gulland (2009), was similar to that described in this study, and will be explored further in Chapter 5.

There are at least three potential alternative explanations for the underlying causes of neck lesions. One is that they are “a normal species-specific feature resulting in [a] post-mortem artefact” (Gulland, 2014; CSG, 2012a). Another is that they could be of traumatic origin, specifically associated with dystocia (CSG 2012a, McLellan 2010 pers. comm.). A third possibility is that they are derived from the effects induced by contaminants, which were passed from the dam to the offspring (McLellan, 2010 pers. comm.).

To further investigate the pathology of these neck lesions, the results of detailed necropsy examinations, including histopathological and microbiological examination, of five fresh calf

cadavers are reported in the next chapter. In addition, in Chapter 5 the potential role of necrotising fasciitis in these lesions is explored and in Chapter 7 the level of contaminants in calves, along with other age groups, is examined.

3.4.1.2.2 Neck lesions in adults

Neck lesions were not found to be associated with dead adults in this study, who were more likely to have evidence of fisheries entanglement. This evidence is in contrast to the suggestions of the CSG (2012a) that trauma from fisheries interaction was contributing to the neck lesions.

To further investigate neck lesions, necropsy examination of three fresh adult cadavers, including histopathological and microbiological examination, is reported in the following chapter (Chapter 4). Furthermore, the data generated from the examination of 13 dolphin carcasses with neck lesions is explored in more detail in Chapter 5, including one adult with a neck lesion.

3.4.2 Study limitations

This study had several limitations because of its retrospective nature.

Firstly, there were no controls used in this study, as it was based on data from dead dolphins. This limits the ability to infer causal associations with mortality; rather, the data describes the distribution of lesions in the presented/found dead animals.

Secondly, the data were the results of studies by a number of observers/researchers and it is likely that there were inconsistencies in the data collection and procedures followed. In order to reduce this potential bias, photographic analysis of necropsied dolphins were used, together with the necropsy reports and histories collected at the time of data retrieval to standardise the data.

Thirdly, some of the changes observed may have been associated with changes post-mortem. In order to limit the potential influence of post-mortem changes, only dolphins of a reasonably "fresh" state (CS2 or 3) were included in the study.

Finally, there were limitations with the comparison study using the photographic data. In the study by McLellan (2010), photographs were not exclusively of carcasses in CS2 or 3, but also included carcasses in CS 4 and 5 which were too decomposed to make a judgement, resulting in a large proportion of carcasses categorised as CNBD. However, comparison of the 69 dolphins with both photographic and gross examination, and the findings with the smaller data set (n=30) found the two methodologies produced similar outcomes.

3.5 Conclusions

This study found evidence that dead dolphins that had signs of fisheries interaction were more likely to be adults than calves, in contrast, dead dolphins with neck lesions were more likely to be calves than adults. In order to reverse the population decline in these dolphins these findings in the different age classes need to be addressed and these will be discussed further in the general discussion in Chapter 9.

As differences were found in the lesions present in adults and calves, it was considered important to undertake a more thorough pathological examination of a small number of cases (n=8) to determine the associated pathology and microbiology, and the results of that study are presented in the following chapter.

CHAPTER FOUR

Pathobiological findings on necropsy examination of eight dead dolphins from 2007-2009, in the Mekong River, Cambodia

4.1 Introduction

In the previous chapter I found that, using existing epidemiological data, the majority of dead adult *O. brevirostris* in the Mekong River was associated with fisheries interaction. In contrast, dead calves were found to display signs of disease and neck lesions. These findings contrasted sharply with unpublished observations by the IUCN Conservation Task Force who wrote that, "*disease, contaminants, and immunosuppression are not contributing to the mortality of the Mekong River Dolphin population*" (Reeves et al., 2009). The findings were, however, in general agreement with those of Schnitzler et al. (2021) who reported that infectious diseases and contaminants were playing a role in mortality of the MRID. To further validate these findings and investigate the potential role of disease in dead MRID in greater detail, I next sought to address a major limitation of the research reported in the previous chapter: that the existing retrospective epidemiological data could not provide information on the underlying disease processes in adult dolphins nor could these data ascertain the pathology of the neck lesions reported among calves.

Previously three studies investigating the pathological changes present in dead *O. brevirostris* have been published, including studies conducted in Vietnam (Yu and Xia, 2013), Malaysia (Kuching, Bintulu-Similajau, Kinabatangan-Segama and Penang Island), India (Chilika Lake), Bangladesh (Sundarbans) (Van Bressem et al., 2014b), and Cambodia (Schnitzler et

al., 2021). Although Van Bressem et al. (2014b) investigated cutaneous lesions; no neck lesions were reported in their study or have been investigated in any of the other published studies. Similarly, a comparison between the disease processes in adults and calves had not been undertaken. Consequently, detailed necropsies on eight dolphins of carcass score 2 (CS2-freshly dead) were undertaken to clearly identify any pathological processes and their potential aetiological role in mortality, and the results of these necropsies are presented in this chapter and their implications for the hypothesised role of disease in the deaths of adult and calf MRID discussed.

4.2 Materials and methods

Necropsy examinations were performed on eight dolphins (three adults and five calves) that died from November 2006 to January 2010. This is a subset of the 41 dolphins listed in Chapter 3. All eight dolphins had a carcass score of 2 (CS2) and were subjected to full necropsies, although only seven had tissue samples submitted for histopathology (Cases 1 - 7), microbiology (Cases 2 - 8) and toxicological analysis (Cases 1-7, Chapter 8) (Table 4-1). One dolphin (Case 8) was necropsied after the samples had been exported from Cambodia to the Netherlands so this case was not available for inclusion in the histopathological analyses. Case 1 was necropsied shortly after I arrived in Cambodia to commence this study, and at this time microbiological testing had not been arranged with the Institut Pasteur du Cambodge, Phnom Penh. Four (50%) of the dolphins (Cases 1, 4, 5, 7) were frozen on the same day as they were found, and subsequently I necropsied them when the Cambodian government authorisation was provided. The remaining four animals (Cases 2, 3, 6, 8) I necropsied within 24 hours of being found.

4.2.1 Necropsy protocol

The necropsies were carried out according to the standardised protocols described by Rowles et al. (2001), which included sample collection for histology, microbiology and cytology. A thorough necropsy was undertaken to detect gross pathological changes and lesions. Photographs were taken of the dead dolphins prior to commencing the necropsies and of any pathological abnormality observed. Samples of lung tissue from all calves necropsied were collected and placed in 10% neutral buffered formalin to assess if the neonate had taken a breath prior to dying (based on whether the lung tissue floated). Skin lesions were defined as tooth rakings if there were two or more parallel lacerations present.

Abnormal findings from each necropsy were categorised and compared with the same age class. The full necropsy protocol template is outlined in Appendix 3 and the report template used for each necropsy is displayed in Appendix 4. Stomach contents were defined as empty in this study if there were no food contents or otoliths present. The presence of sand sediment in the stomach chambers was noted.

4.2.2 Microbiological sample collection

Any gross pathological abnormalities were aseptically swabbed using an Amies agar gel sterile transport swab (Thermo Fisher Scientific Australia Pty Ltd). Priority was placed on obtaining samples from the cervical region and/or neck lesions of all dolphins necropsied. The necks of all carcasses were initially scrubbed with 7.5% povidone-iodine (Betadine®) surgical scrub, followed by a 95% alcohol scrub. Then an incision was made with a sterile 22G scalpel blade and a full thickness (4cm x 4cm - approximately 50g) sample of neck

blubber, muscle and fascia was collected and placed in a sterile container, which was subsequently refrigerated at 4°C.

Internal organs were similarly sampled while they were *in situ*, immediately after opening the body cavities, and approximately 50g placed in a sterile container, which was subsequently also refrigerated at 4°C. Samples were taken of the liver, kidney, lung, aorta with blood, as well as any other tissue/organ displaying gross pathological changes. In addition, serosanguinous fluid in the thorax and/or abdomen, amniotic fluid, and urine (if present) were collected aseptically using a sterile needle and 20mL syringe.

In total, 83 tissues and organ samples from seven dolphins (Cases 2-8) were collected for microbiology. These samples were transported on ice to the Institut Pasteur du Cambodge in Phnom Penh to Dr Bertrand Guillard for microbiological culture under aerobic and anaerobic conditions using standard protocols as described in Schnitzler et al. (2021). No samples from this study were cultured for viruses due to the inability to store tissues at -80˚C, or to isolate viruses in Cambodia during the time this study was undertaken.

4.2.3 Cytology

Cytological preparations of all tissue samples and swabs collected, including impression smears, were stained using Diff-Quik stain (RAL Diff-Quik™), consisting of a fixative agent (methanol), solution I (eosinophilic) and solution II (basophilic). Slides were dipped sequentially into each solution for 15 seconds, followed by a water rinse and left to dry. These slides were sent together with the tissues collected for microbiological testing to the Institut Pasteur du Cambodge. Cytology results for leucocytes (not-categorised) were coded

by the laboratory as: absent (0), rare (1+), some (2+), numerous (3+), and very numerous (4+).

In selected cases where masses/growths were found, I performed in-house cytology (impression smears and fine needle aspirate cytology) immediately after the necropsy. These were carried out using the Diff-Quik stain as described above. Slides were examined using a Premiere MSB-02 Binocular Microscope, with an HP X 100 oil immersion lens.

4.2.4 Histology

Samples were collected for histopathology as outlined in Appendix 3 and fixed in 10% neutral buffered formalin. Two specimen jars were labelled for each carcass: one for the samples of all tissues which appeared normal, and one for the samples of tissues with suspected pathology. Routine samples collected included: blubber, tongue, oesophagus, larynx, trachea, lung, heart, aorta, pulmonary artery, liver, stomach (3 chambers), small intestine (duodenum, ileum), large intestine (colon), uterus, ovaries, testes, adrenal glands, kidneys, brain, spinal cord, skin, muscle, diaphragm, melon, spleen, bladder, several lymph nodes, and thymus. All formalin fixed samples were sent to the Department of Viroscience, Erasmus University Medical Centre, Rotterdam, Netherlands. These samples were further prepared for histopathological examination according to standard protocols. Briefly they were embedded in paraffin, and 5µm sections were cut and stained with haematoxylin and eosin (H & E) as described by Schnitzler et al. (2021).

Histological examination was kindly performed by Prof. Thijs Kuiken, Professor of Comparative Pathology, Erasmus University Medical Centre who also provided a

morphological diagnosis and ranking based on the lesion's severity (mild, moderate, severe), duration (acute, sub-acute, chronic), distribution (diffuse, multifocal, zonal), and pathological process (inflammation, neoplasia, necrosis and a modifier: fibrinous, granulomatous, necrotising or proliferative**)**.

4.2.5 Statistical analyses

The abnormal findings from the eight necropsies were categorised into two main groups: pathological processes; and evidence of trauma, in order to see if any common pathological findings emerged across cases and dolphin age categories. *Robvis* (McGuinness and Higgins, 2020) was used to create traffic-light plots of the data. The Fisher's exact test was used to determine significant differences in the pathological presentations between adults and calves using the Real Statistics Resource Pack (Release 5.4) for Excel (2010) (Zaiontz, 2018).

4.2.6 Ethics approval

Animal ethics approval to use and collect samples from cadavers was obtained from Murdoch University for this study. Permission was also granted to conduct the necropsies by the Kingdom of Cambodia through the Ministers associated with the Fisheries Administration (MAFF) and the Dolphin Commission (DC).

4.3 Results

The five calves weighed between 3.8 and 17Kg and the three adults between 102 and 110Kg (Table 4-1). Data of the gross abnormalities detected and trauma present in the eight dolphins necropsied are summarised in Figure 4-1 and Figure 4-2, respectively.

Table 4-1: Summary data of dolphins necropsied with gross pathological changes observed and tests conducted

Case #	ID #	Specimen number	Age	Length (m)	Weight (kg)	Sex	Date collected	Date necropsied	Microbiology & cytology	Histology
Case 1	71	OBRE0609/12*	Calf (N)	1.09	12.5	F	09/12/06	10/12/06	✕	✓
Case 2	80	CID 07 009	Calf (N)	0.93	9.0	M	10/04/07	10/04/07	✓	✓
Case 3	83	CID 07 012	Calf (N)	1.07	17.0	F	13/07/07	13/07/07	✓	✓
Case 4	85	CID 07 014*	Calf (N)	0.69	3.8	F	31/12/07	16/01/08	✓	✓
Case 5	88	CID 08 001*	Calf (N)	1.04	14.5	F	02/01/08	16/01/08	✓	✓
Case 6	92	CID 09 002	Adult	2.18	110.0	F	08/01/09	08/01/09	✓	✓
Case 7	96	CID 09 008*	Adult	2.2	106.0	M	09/08/09	31/10/09	✓	✓
Case 8	102	CID 10 002	Adult	2.30	102.0	F	23/01/10	23/01/10	✓	✕

* Carcass frozen prior to necropsy examination; ID #: Identification number; Calf (N): Newborn Calf; ✓ procedure conducted; ✕ procedure not conducted.

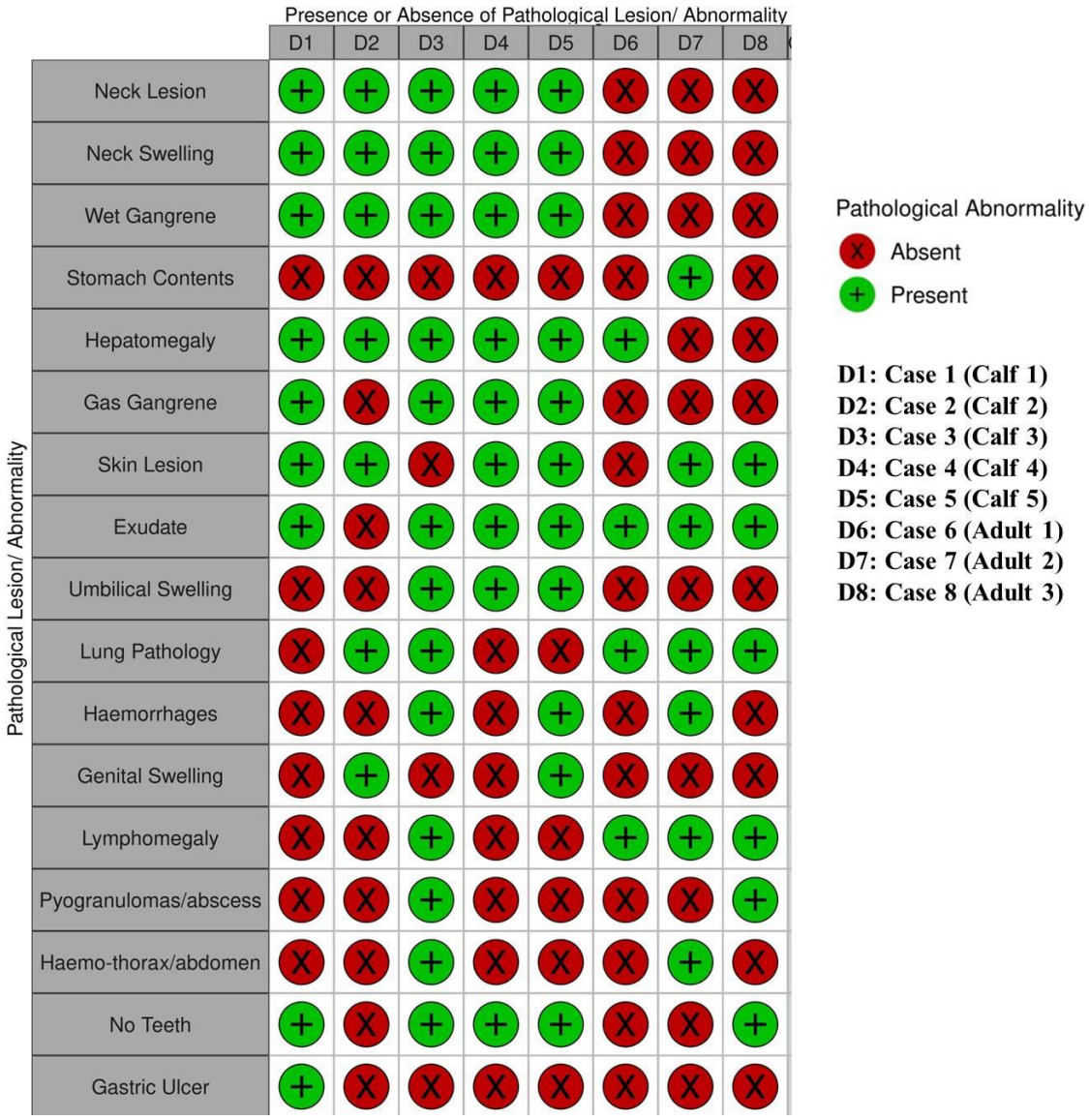


Figure 4-1: Presence and absence of gross pathological lesions and abnormalities in the eight necropsied dolphins.

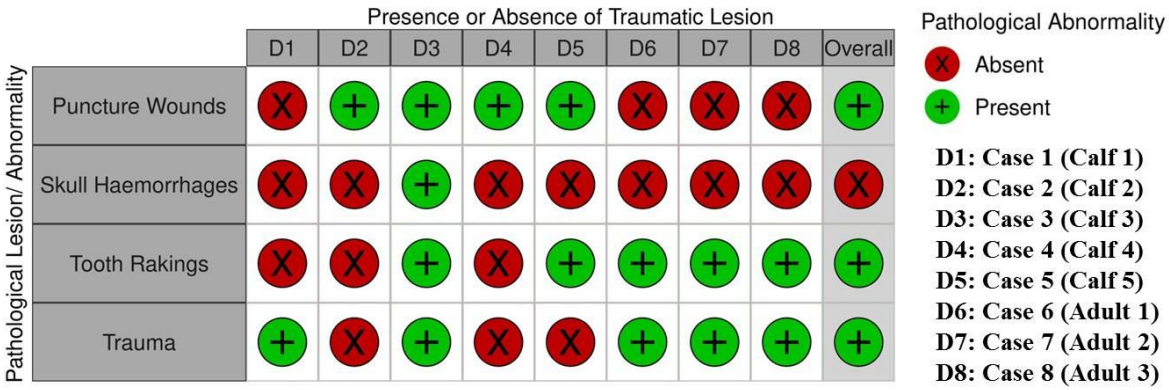


Figure 4-2: Summary of evidence of trauma in the eight necropsy cases.

A summary of the key necropsy findings, including gross pathological changes and histological and microbiological findings, in each of the eight cases is presented below.

4.3.1 Case 1 - Calf 1

4.3.1.1 External examination

The dolphin calf had labial hairs; however, the umbilical cord was absent. Bruising was evident on the ventral cervical area, extending from the mandible to the pectoral flippers and down the right pectoral flipper. The ventral cervical region appeared swollen (Figure 4-3). Gas was detected in a fine needle aspirate (FNA) of this swelling. There was a “tattoo-like” lesion on the right ventral neck region. There was also a small amount of serosanguinous fluid in the blowhole.

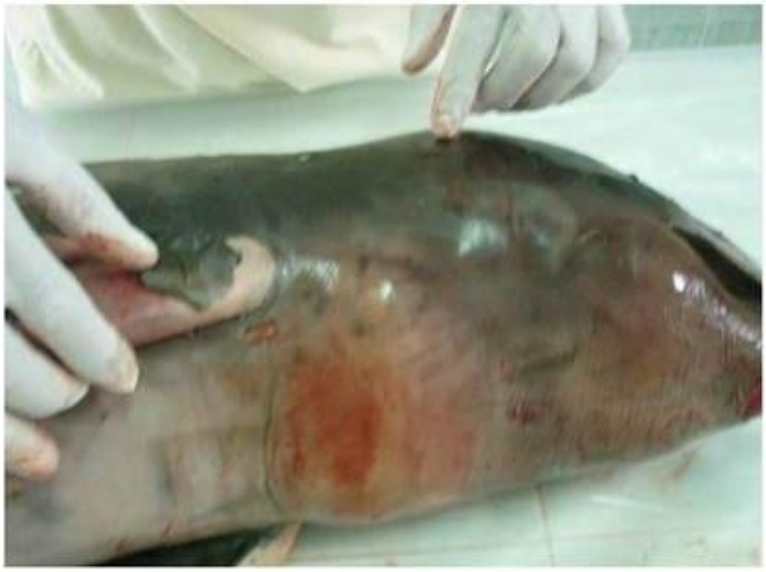


Figure 4-3: Neck lesion in Case 1.

4.3.1.2 Internal examination

The musculature on the ventral aspect of the rostrum, neck and pectoral areas contained gas-filled pockets with a black, wet appearance. This was in contrast to the normal pink musculature on the dorsum of the same area, and on the ventral abdomen. The 8th right rib displayed signs of an ante-mortem fracture, with evidence of bruised tissue in the region of the broken rib.

A sample of lung tissue floated in formalin, indicating the calf had breathed prior to death. Ulcerative lesions were present on the tongue. The third gastric chamber and duodenum both contained a small amount of fine sand. Gastric mucosal ulcerations (multifocal lesions approximately 1cm in diameter) were evident in the second gastric chamber. Histologically, the gastric submucosa was infiltrated by moderate numbers of inflammatory cells, confirming multifocal gastric ulceration (Kuiken 2010, pers. comm.). The liver had rounded margins and was very firm with a rubbery

consistency. Its architecture appeared to be structurally intact with few autolytic changes.

4.3.2 Case 2 - Calf 2

4.3.2.1 External examination

The calf carcass had foetal folds, with tooth buds on both the maxilla and mandible. No labial hairs were present. There was a large swelling on the ventral neck region, with a slight dark blue/black discolouration. Two puncture lesions were apparent bilaterally on the lateral aspect of the neck, separated by a distance of 11cm. Each lesion was irregular in shape, but appeared to have two rounded areas within both lesions. The puncture wounds extended through the epidermis into the blubber. The right lesion was 3cm long and 1cm wide and the left lesion 2.5cm long and 1cm wide. Both lesions were 15cm from the snout.

Four “tattoo-like” skin lesions were present on the dermis and epidermis - two on the left lateral aspect below the dorsal fin, one on the left side of the head and one on the left fluke (Figure 4-4). Under the epidermis on the left dorsum and lateral aspect there were several multifocal black irregular areas of discolouration, with an indented stippled appearance (Figure 4-4). Histologically, the lesions consisted of moderate numbers of inflammatory cells (type not specified) and bacterial infiltrates, confirming multifocal skin ulceration (Kuiken, 2010 pers. comm.).

There was no serosanguinous fluid present in any orifice.

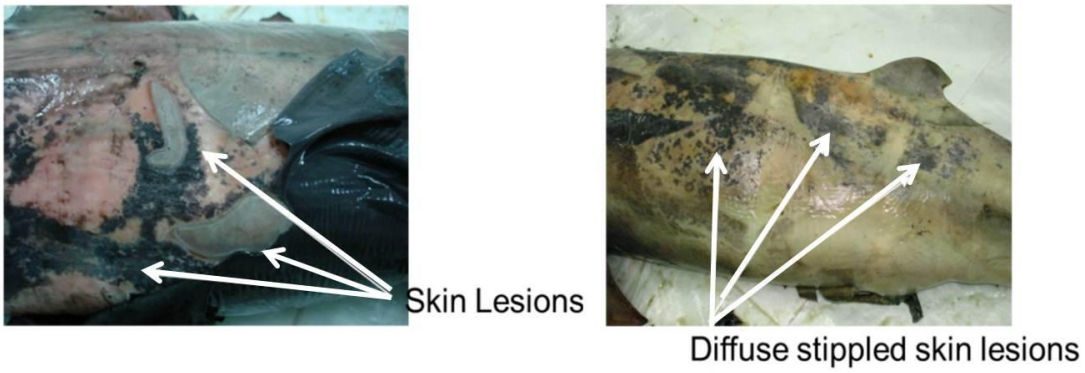


Figure 4-4: Tattoo-like skin lesions in Case 2.

4.3.2.2 Internal examination

The musculature in the ventral cervical region extended from the snout to the flippers and had a very wet exudative blackened appearance. There was an area of blue/black discolouration on the peduncle on the left side. Similar to the muscles in the neck, the musculature of the peduncle on the right lateral aspect was slightly black in colour. No gas was found.

The lungs appeared congested and consolidated. They contained very little air. Samples from both lung lobes initially sunk in formalin, although they floated within 3 minutes of being placed in it. There was diffuse hepatomegaly with very rounded margins and a rubbery texture; however, on histological examination the samples were too autolysed for interpretation (Kuiken, 2010 pers. comm.). All stomach chambers were empty.

Numerous colonies of *Aeromonas hydrophila* and *Morganella morganii* were cultured from all specimens submitted, including from the aorta, neck lesion, kidney, lung, liver, neck muscle and blubber, and a blowhole swab.

4.3.3 Case 3 - Calf 3

4.3.3.1 External examination

The calf carcass had foetal folds present; however, there were no labial hairs and the teeth had not yet erupted. There was slight skin sloughing, a swollen tongue, and the ventral neck region was also swollen.

There were recent parallel lacerations on the left lateral side of the body. The underlying tissue around the lacerations appeared erythematous. The right side of the head had two full thickness skin wounds that appeared to be older wounds. There was significant recent bruising (erythematous colouration) on the ventral region of the neck, on the dorsal aspect of the head and on the peduncle just cranial to the fluke (Figure 4-5). There were generalised petechial haemorrhages throughout the epidermis and on the dermis, as well as on some underlying muscles (Figure 4-6). In addition, there were also localised areas with larger ecchymotic haemorrhages. Approximately six ecchymotic haemorrhages were present on the epidermis on the right lateral side of the carcass. There were approximately four darker areas of discolouration on the erythematous head region.

The umbilicus was very swollen and had serosanguinous exudate leaking from it (omphalitis). A serosanguinous exudate was also detected bilaterally from the aural orifices. Bilateral exophthalmos was evident.

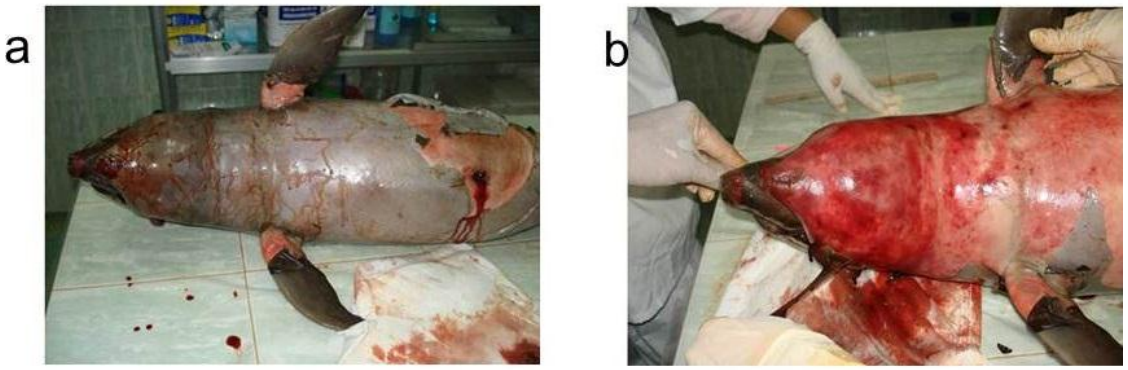


Figure 4-5: Case 3, highlighting the extent of the neck lesion a) darker swollen area on ventral cervical region, b) on removal of the epidermis an extensive area of haemorrhagic appearance was present around the cervical and head regions.

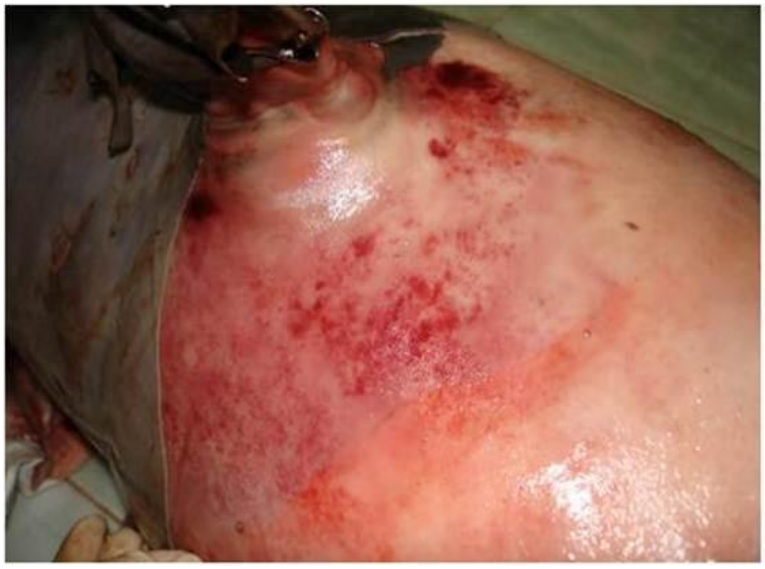


Figure 4-6: Case 3 - dolphin calf with diffuse pathology and omphalitis with serosanguinous exudate and presence of ecchymotic haemorrhages.

4.3.3.2 Internal examination

The muscles under the mandible were black, wet and oozing with gas bubble formation. Similar lesions of the muscle were seen bilaterally on the side of the head, over the left scapula, and on the dorsal aspect of the head.

The left lung had 10 pyogranulomatous caseous lesions throughout the lobe, with the largest one measuring 5cm in diameter (Figure 4-7). The right lung lobe had a diffuse, stippled, whitish appearance on the caudal quarter, and had three large areas of what appeared to be haemorrhages, covering approximately 2/3rds of the lobe. Tissue samples from both lobes floated in formalin. *Plesiomonas shigelloides* and *A. hydrophila* were cultured from the pyogranulomatous lesions. Histologically, a diagnosis of bronchopneumonia (pyogranulomatous, necrotising, diffuse, chronic, marked) was confirmed by Kuiken (2010 pers. comm.). The alveolar lumens were diffusely filled with many degenerate neutrophils and fewer alveolar macrophages, mixed with fibrin and erythrocytes. The alveolar walls were infiltrated with moderate numbers of neutrophils, and many alveolar capillaries were congested and contained fibrinous thrombi. The bronchiolar and bronchial lumens contained many neutrophils, and the walls were infiltrated with moderate numbers of neutrophils.

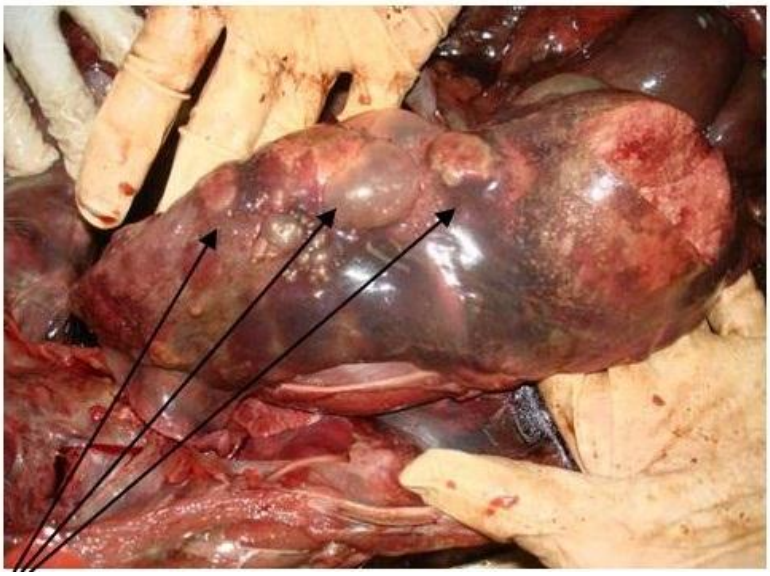


Figure 4-7: Case 3 - lung lobe with several pyogranulomatous caseous masses (arrows) from which *A. hydrophila* and *P. shigelloides* were cultured.

A haemothorax was present with 82mL of serosanguinous fluid collected from the thoracic cavity. The thoracic lymph nodes were enlarged. Pyogranulomatous lesions were present on the major vessels extending from the base of the heart. The heart appeared normal; however, the blood within the ventricles had not clotted. Hepatomegaly was present with diffuse rounded margins and a rubbery texture. Histologically, there was fibrin present in the liver.

There was a small amount of sand in the oesophagus and in the first three stomach chambers; however, there were no other stomach contents.

4.3.4 Case 4 - Calf 4

4.3.4.1 External examination

This carcass was very small, weighing approximately one-third of the normal weight and two-thirds of the normal length of a calf (90-110cm, (Beasley, 2007)). Labial hairs were present, although no teeth had erupted. The umbilical cord appeared to have been recently severed, resulting in an open wound (omphalitis)

There was a very prominent blue/black lesion on the ventral neck region (Figure 4-8). Numerous penetrating skin lesions were present, as well as generalised multifocal skin indentations/punctate lesions (Figure 4-9). On histological examination, Kuiken (2010, pers. comm.) found two medium-sized aggregates of inflammatory cells, with lymphocytes and plasma cells, and a few macrophages at the edge of the blubber layer. This was diagnosed as multifocal, mild, chronic, lymphocytic panniculitis of the blubber.

Figure 4-8: Case 4 - neck lesion clearly visible on the carcass.

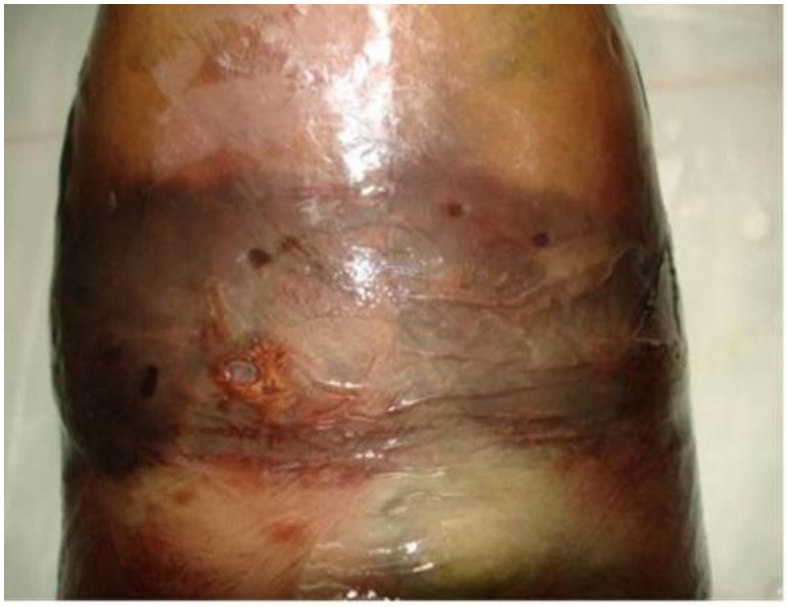


Figure 4-9: Case 4 – evidence of penetrating lesions on the ventral cervical skin.

4.3.4.2 Internal examination

When the epidermis was removed from the carcass, notable discolouration was present on the head, with a distinct demarcation from the normal musculature

caudally (Figure 4-10). The musculature on the ventral cervical region, and the upper pectoral area was blue/black, wet, oedematous and gaseous in nature.

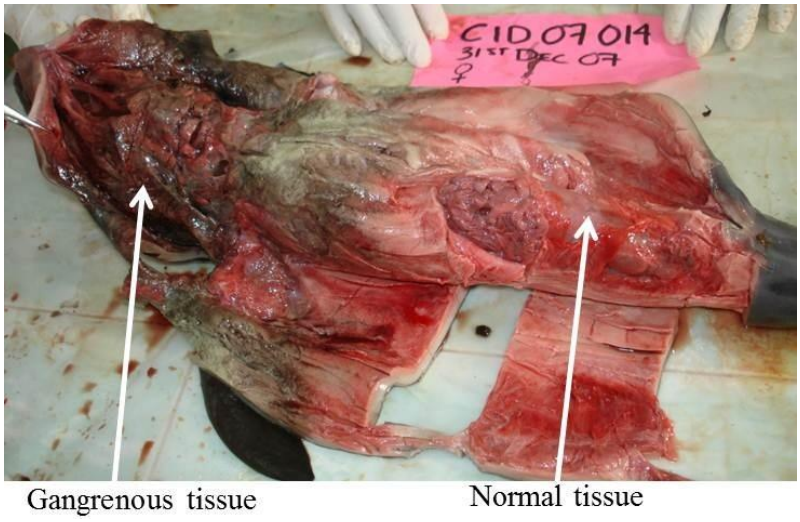


Figure 4-10: Case 4 - musculature of the entire body exposed showing very dark gangrenous discolouration to the head and cervical area, with normal musculature caudally on the abdomen and peduncle.

There was a small amount of frothy exudate present in the blowhole that did not extend caudally into the larynx or bronchi. Samples from both lung lobes floated in formalin. The liver appeared grossly enlarged with rounded margins. All stomach chambers were empty. The large colon was filled with thick brown faecal matter (meconium).

Numerous colonies of *P. shigelloides* were cultured from the neck lesion, lungs, blood, kidney and umbilicus.

4.3.5 Case 5 - Calf 5

4.3.5.1 External examination

The carcass had foetal folds and labial hairs present, and the teeth had not erupted.

Blue/black discolouration on the ventral neck region was prominent and the cervical region appeared swollen (Figure 4-11). There were several gas filled vesicles on the skin on the right lateral side and on the flukes. There were numerous penetrating skin lesions around the body, as well as parallel linear skin lesions. The genital region appeared swollen and the umbilicus had serosanguinous exudate discharging from it (Figure 4-12).

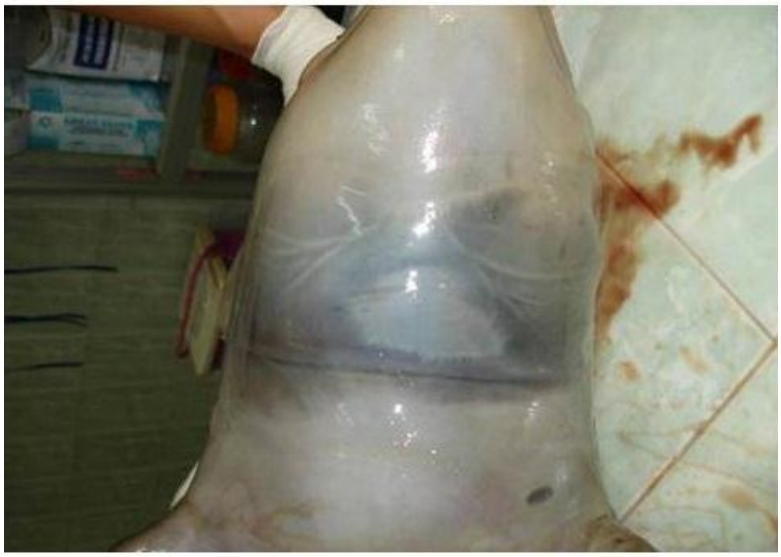


Figure 4-11: Case 5 - ventral neck lesion visible on the carcass.

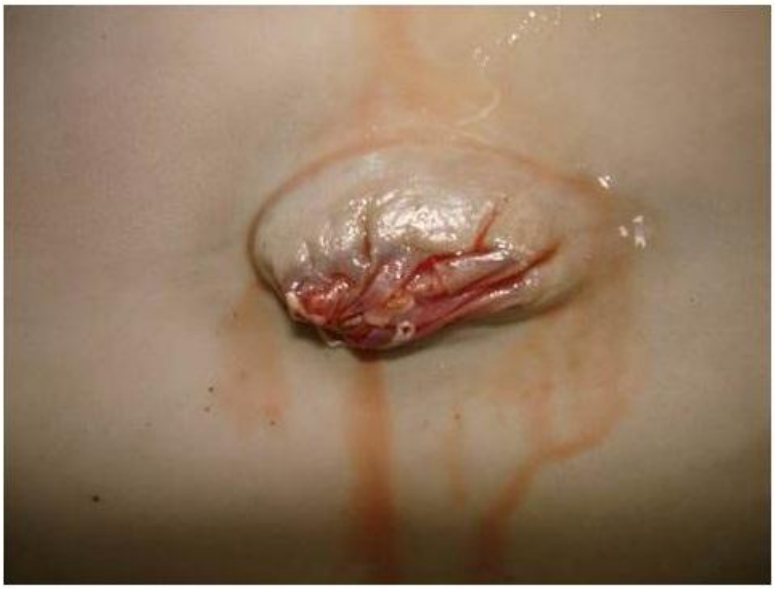


Figure 4-12: Case 5 - umbilicus with serosanguinous discharge.

4.3.5.2 Internal examination

When the epidermis was removed from the carcass, discolouration to the head and cervical area was apparent. Petechial haemorrhages were present on the peduncle and the tail stock. On cut sections of the ventral neck region there was blue/black wet oedematous and gaseous musculature extending past the pectoral area. In contrast, musculature caudal to the cervical region was normal, with a distinct demarcation between the affected and unaffected musculature. Numerous white multifocal small areas were found in the musculature of the cervical region. Histologically, the only abnormality observed by Kuiken (2010, pers. comm.) were abundant lymphocytes around the submucosal glands of the laryngeal wall.

The lungs were firm and rubbery and samples of both lobes floated in formalin. Numerous white multifocal small areas were found on the pericardium and the heart.

These were also present on the diaphragm and the thoracic and abdominal pleura and on the liver, as well as within the neck muscle (Figure 4-13).

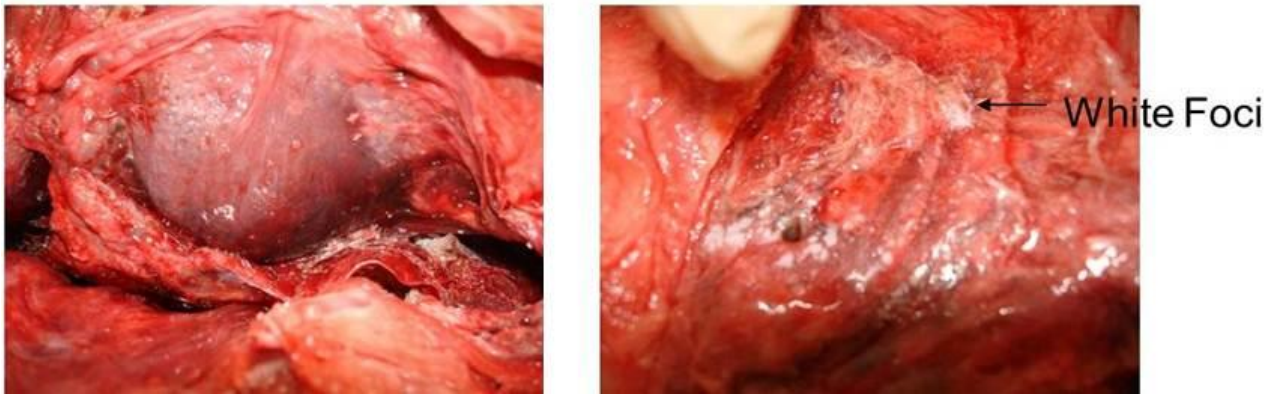


Figure 4-13: Case 5 - generalised distribution of multifocal white plaques.

The liver appeared grossly enlarged, with distended round margins, and had a rubbery consistency. The stomach chambers were empty, other than for a small amount of sand in the first chamber. The colon was filled with dark brown faecal material (meconium).

Plesiomonas shigelloides were cultured from the neck lesion, lung, blood, kidney, liver and white plaques.

4.3.6 Case 6 - Adult 1

4.3.6.1 External examination

This adult dolphin was initially found alive with head injuries, but died shortly after discovery, and was necropsied on the same day. The carcass was emaciated, with vertebral processes palpable. Only three teeth were present and these were severely

worn down, presumably by dental attrition. There were no neck lesions or discoloured musculature present on the carcass; however there were several head lacerations (Figure 4-14). Numerous rods and cocci were seen on histological examination in the exposed dermis of these lacerations (Kuiken 2010, pers. comm.). There was diffuse infiltration with moderate numbers of neutrophils in the underlying dermis, together with areas of focal haemorrhage. The skin lesions were diagnosed histologically as dermatitis (multifocal, ulcerative, acute and mild). The lesions in the skin were consistent with superficial lacerations with secondary bacterial infection (Kuiken 2010, pers. comm.).

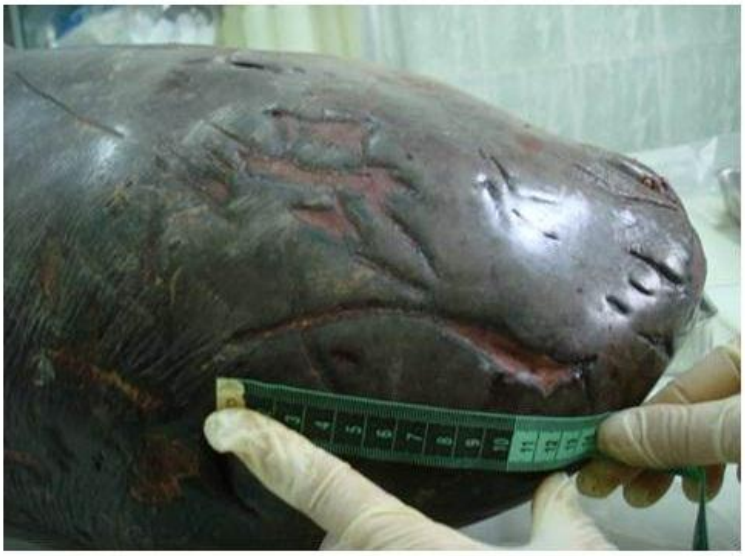


Figure 4-14: Case 6 - skin lacerations on the head region.

4.3.6.2 Internal examination

The lungs, trachea and bronchi were full of serosanguinous fluid and frothy exudate. The left lung lobe had a diffuse pitted/moth-eaten appearance grossly extending over half of the cranial aspect of the lobe. Palpably, both right and left lung lobes had a

generalised distribution of thickened tissue masses, and on incision these appeared to be thickened bronchi and bronchioles (Figure 4-15). Within the lumen of the alveoli and bronchioles moderate numbers of neutrophils and a few macrophages were detected on histology and a diagnosis of bronchopneumonia (suppurative, multifocal, acute, moderate) was made by Kuiken (2010, pers. comm.).

Numerous neoplastic-like extrapulmonary masses were found in the thoracic cavity and on sectioning they appeared to be lymphoid tissue, with several of these masses containing black pigment. A FNA of one of these lymph nodes demonstrated both large and small lymphocytes, the occasional round cell with a large amount of cytoplasm, erythrocytes and the occasional monocyte present. Examination of an impression smear of this lymph node confirmed the presence of round cells with multiple nuclei. Histologically, these were identified as macrophages with black carbon pigmentation (anthracosis), and a diagnosis of lymph node anthracosis was made (Kuiken 2010, pers. comm.). In addition, neutrophils and lymphocytes with normal morphology were present.

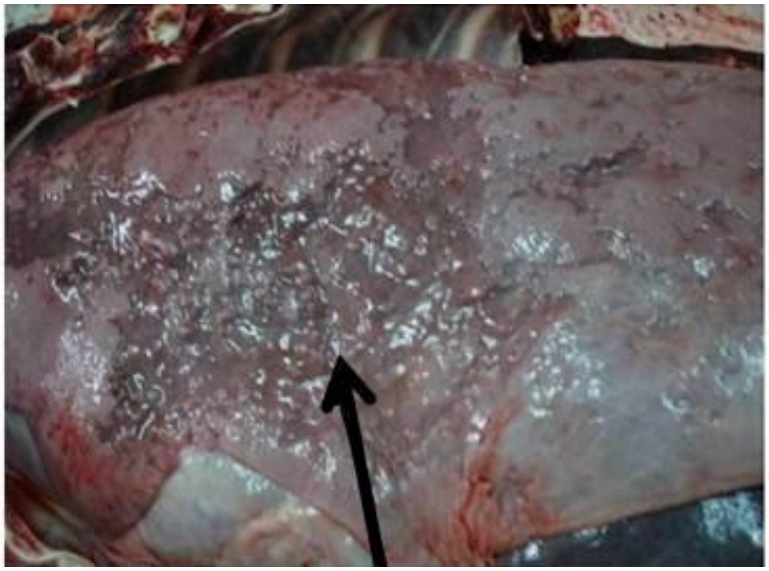


Figure 4-15: Case 6 - gross lung pathology.

The liver appeared grossly raised and rubbery. Histologically, Kuiken (2010 pers. comm.) found evidence of diffuse hepatic lipidosis and peri-portal hepatic atrophy. Occasional white surface nodules on the liver, with a white/yellow creamy exudate on incision, were observed. *Aeromonas hydrophila* was cultured from this exudate.

Histological analysis of the spleen revealed mildly hypocellular lymphoid follicles with no evidence of apoptosis.

The heart was grossly distended, with the right ventricle very dilated with a hypoplastic wall (0.5cm in thickness). In contrast, the left ventricular wall was markedly hyperplastic (2.5cm in thickness). Indicating this dolphin had hypertrophic cardiomyopathy. Additionally, the aorta was grossly distended and enlarged.

The alimentary tract was empty of food contents, although sand was found in the first three stomach chambers. The kidneys showed no gross pathology, although on histological examination multifocal, mild renal tubular mineralisation was detected. Howell–Jolly bodies were present in approximately 5% of the RBCs.

Aeromonas hydrophila was cultured from multiple organs and pathological sites in this dolphin.

4.3.7 Case 7 - Adult 2

4.3.7.1 External examination

This carcass had generalised dental wear (attrition). Several teeth were protruding through the lower jaw (five on the left mandible, two on the right mandible), and exiting ventrally to the gape (Figure 4-16). Numerous lacerations were present on the body, including some parallel lacerations (Figure 4-17). Several dark pigmented lesions, with a generalised multifocal distribution, were found over the head and body extending through the dermis and epidermis (Figure 4-18). The epidermis of the skin lesion was hyperplastic being approximately 1.5 times thicker than normal epidermis, and Kuiken (2010 pers. comm.) diagnosed epidermal hyperplasia that was moderate and focal to diffuse.

Figure 4-16: Case 7 - dental malformation with several teeth protruding through the mandible.

Figure 4-17: Case 7 - heavily scarred body.

Figure 4-18: Case 7 - dark irregular pigmented skin lesions with a multifocal generalised distribution over the body.

4.3.7.2 Internal examination

There were no obvious neck lesions present in this dolphin. There were several multifocal haemorrhagic foci within cut sections of blubber on the dorsolateral aspect of the carcass just ventral to the dorsal fin (Figure 4-19) along with epidermal hyperplasia. Bradyzoites of *Sarcocystis* spp. were detected histologically in both the external abdominal oblique muscle and samples submitted from the cervical muscles (Kuiken 2010, pers. comm.).

Brown frothy exudate was found in the trachea, bronchi and on cut sections of the lung lobes suggesting pulmonary oedema. The pulmonary lymph nodes were enlarged. The thoracic and abdominal cavities contained >500 ml of sterile serosanguinous exudate.

Figure 4-19: Case 7 - generalised multifocal haemorrhages within the dermal blubber layer.

The stomach was almost empty. The first chamber had a small volume of soft grey exudate, the second chamber had numerous yellow hard micro beads 3mm in diameter, and the third chamber had a small volume of sand.

The brain appeared grossly normal; however on histology Kuiken (2010 pers. comm.) detected the presence of a few neutrophils in the pia mater in the pons, and the medulla oblongata, and a moderate number of neutrophils in the cerebrum. A diagnosis of suppurative bacterial leptomeningitis (acute, diffuse and severe) was made. Unfortunately cultures were not submitted from the brain tissue.

Several microorganisms were cultured from this dolphin from different sites: non-*albicans Candida* spp. (blowhole); *Klebsiella pneumoniae* (kidney, muscle, liver); *Citrobacter freundii* (kidney, neck muscle); *β*-haemolytic Group D *Streptococcus* spp. (neck muscle); *E. coli* (neck muscle, muscle, liver); and *Enterobacter aerogenes* (neck muscle).

On cystocentesis, haematuria (17 mm^3), crystaluria (calcium oxalate), leucocyturia (14 mm^3), proteinuria (3+) and bacteriuria (*Enterobacter cloacae*) were detected, and a diagnosis of cystitis was made.

4.3.8 Case 8 – Adult 3

4.3.8.1 External examination

This dolphin was observed by villagers floating on the surface of the water and moving in circles. She died before the research team arrived and the necropsy commenced within two hours of death. Within 15 minutes of her death Cambodian Government fisheries officers reported there was serosanguinous exudate pooling from her mouth.

The carcass was emaciated with pronounced peduncle neural spinous processes, protruding chevron bones (Figure 4-20) and noticeable depletion of fat stores around the head area. Teeth were absent, with evidence of teeth having protruded through the mandible, exiting below the gape. The abdomen appeared notably distended, with uterine membranes protruding through the genital aperture, suggesting a gravid female, which was confirmed on opening the abdomen. This carcass had several linear parallel lacerations in a generalised distribution on the body, with recent/fresh lacerations evident on the head and ventral thoracic region. The carcass had a circular dark band of discolouration around the neck region (Figure 4-21). There was a generalised multifocal distribution of dark pigmented “tattoo-like” skin lesions over the body (Figure 4-22).

Figure 4-20: Case 8 - lateral view of the emaciated body condition with several chevron bones/transverse processes visible on the peduncle (arrow).

Figure 4-21: Case 8 - dark band of pigmentation (arrow) around the neck.

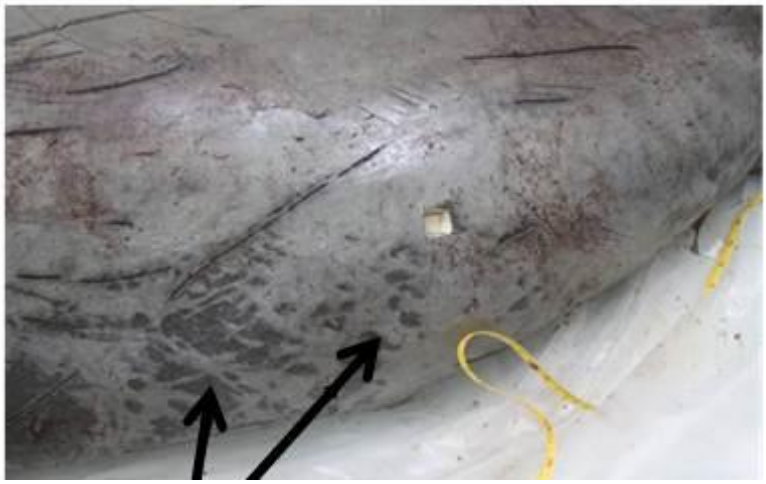


Figure 4-22: Case 8 - "Tattoo-like" skin lesions with a multifocal generalised distribution over the body (arrows).

4.3.8.2 Internal examination

Cut sections of the ventral cervical musculature haemorrhaged profusely, and were considered to be caused by post-mortem lividity. Petechial haemorrhages were noted throughout the blubber layer.

This dolphin was found to be gravid with apparently normal twin foetuses, with two amniotic sacs. The first foetus (CID 10 003) weighed 430g and was 26cm long, and the second foetus (CID 10 004) weighed 530g and had a total length of 34cm. 30mL of pink amniotic fluid was collected aseptically via amniocentesis. Pure cultures of *A. hydrophila* and *β*-haemolytic Group C *Streptococcus* spp. were cultured from this amniotic fluid, indicating chorioamnionitis.

The lungs had a generalised mottled appearance with calcification. On cut sections there were demarcated areas of pale-pink necrotic tissue on the surface of the lung

extending 1cm into the parenchyma. A small amount of frothy pink exudate was present on the cut sections of the lungs, in the trachea and in both bronchi.

This dolphin had a large (19cm) well-circumscribed pyogranulomatous abdominal mass with a neoplastic appearance, with several darker well circumscribed nodules within the capsule of the mass (Figure 4-23), in association with mesenteric lymphadenomegaly. This mass was adhered to the caudal margin of the stomach and the left adrenal gland, and the mesentery of the small intestines. The left adrenal gland and the spleen were incorporated within this larger pyogranulomatous mass. On excision the mass contained thick, pale-green coloured, exudative material with a lumpy appearance and was diagnosed as a splenic abscess. The thickened wall of the mass also contained hardened white areas of tissue resembling cartilage. A second 5cm well-circumscribed cystic mass was found below, but adhered to, the larger mass. This second mass had a very thick cartilaginous capsule, and on excision was hollow and contained dark, thick, serosanguinous exudate.

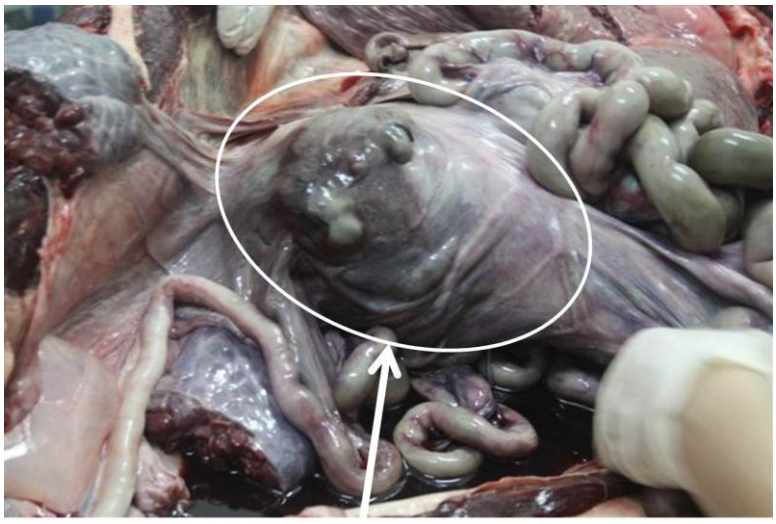


Figure 4-23: Case 8 - pyogranulomatous neoplastic-like splenic mass (arrow).

The heart appeared normal; however the pericardial sac was notably thickened. The stomach had some dark brown exudate and contained sand. The contents of the small intestine included a light green coloured exudate and the contents of the colon a light brown coloured exudate.

Unfortunately samples from this animal were not available for histopathological testing. Analysis of urine collected via cystocentesis revealed haematuria (92 mm^3 - reference level (Ref) <10 (Bertrand Guillard pers. comm.), leucocyturia (23 mm^3, Ref <10 (Bertrand Guillard pers. comm.)), bacteriuria (*A. hydrophila*) and crystaluria (calcium oxalate), confirming a urinary tract infection (UTI).

Plesiomonas shigelloides, *A. hydrophila* and *β*-haemolytic Group C *Streptococcus* spp. were cultured from the pyogranulomatous exudates. *Aeromonas hydrophila* and *β*-haemolytic Group C *Streptococcus* spp. were also cultured from all major organs, uterus, blowhole, blood and muscle of this animal.

4.3.9 Analysis of the necropsy findings from the eight cadavers

When the results from the eight dolphins in this study were subdivided into age class, significantly more calves 100% (95%CI 47.8, 100) had neck lesions and associated neck swelling with wet gangrenous musculature than adults (0%; 95%CI 0.0, 70.8) (Fisher's exact test p=0.018). There were no significant differences between adults and calves for all other necropsy findings (all $p \geq 0.07$). All five calves had empty stomachs other than sand, and hepatomegaly; however this was only found in one

adult case (Case 7) (p=0.11). Three calves (Cases 1, 3, 5) (60%; 95%CI 14.7, 94.7) had a small amount of sand/sediment in the alimentary tract compared to all three adults (p=0.46). Four calves (80%; 95%CI 28.4, 99.5) had puncture wounds compared to no adults (p=0.07), however all adults had tooth rakings (100%; 95%CI 29.2, 100), compared to only two of the calves (40%; 95%CI 5.3, 85.3) (p=0.18). All three adults necropsied had serosanguinous exudate, evidence of trauma, and lymphomegaly (100%; 95%CI 29.2, 100).

In this study, two of the three adults necropsied were confirmed to have urinary tract infections. One adult had no teeth, and another only had three teeth remaining, suggestive of aged animals.

Three calves (Cases 3, 4, 5) had omphalitis. Three also had labial hairs (Cases 1, 4, 5) and two (Cases 4, 5) had meconium, which suggests that these animals were newborn calves which died shortly after birth. Only one calf had tooth buds (Case 2), suggesting a slightly older newborn calf. Additionally, three calves had foetal folds (Calf 2, 3, 5) present suggesting they were newborn animals.

4.3.9.1 Microbiological and cytological analysis

In Table 4-2 the cytology and microbiological culture results from the Institut Pasteur du Cambodge, Phnom Penh are presented for seven cases (no samples from Case 1 were submitted for culture). *Aeromonas hydrophila* were isolated from 45% (n=37 samples from 4 dolphins) of the 83 samples cultured, *P. shigelloides* from 27% (n=22

from 4 dolphins) of the samples, and *β*-haemolytic Group C *Streptococcus* spp. from 12% (n=10 from 2 dolphins) of the samples.

Of the 83 samples examined, 51 had cytological grades from 0 to 4+ (3 (6%), 11 (22%), 16 (31%), 2 (4%) and 19 (37%) had scores 0, 1+, 2+, 3+ and 4+, respectively) (Table 4-2).

The microbiological culture results from the Institut Pasteur du Cambodge Laboratory, Phnom Penh, together with the histopathological results provided by Kuiken (2010, pers. comm.), are presented for the seven cases submitted for testing in Appendix 5.

Table 4-2: Microorganisms isolated and associated cytology from seven necropsied Mekong River Irrawaddy dolphins

Case no	ID #	Positive samples	Sample origin	Cytology: leucocytes grade[#] / measure (mm^3)	Microorganisms	Colony growth score *
2	CID 07 009	9	Neck lesion	1+	*Aeromonas hydrophila*	3
					Morganella morganii	3
			Muscle	2+	*A. hydrophila*	3
					M. morganii	3
			Lungs	1+	*A. hydrophila*	3
					M. morganii	3
			Kidney	4+	*A. hydrophila*	3
					M. morganii	3
			Liver	1+	*A. hydrophila*	3
					M. morganii	3
			Blood	1+	*A. hydrophila*	3
					M. morganii	3
			Blow hole	1+	*A. hydrophila*	3
					M. morganii	3
			Serosanguinous exudate	19.8mm3	*A. hydrophila*	3

Case no	ID #	Positive samples	Sample origin	Cytology: leucocytes grade[#] / measure (mm^3)	Microorganisms	Colony growth score *
3	CID 07 012	5	Neck lesion	2+	*A. hydrophila*	3
					Plesiomonas shigelloides	3
			Muscle	2+	*A. hydrophila*	3
					P. shigelloides	3
			Lungs	4+	*A. hydrophila*	3
					P. shigelloides	3
			Aorta	1+	*A. hydrophila*	3
					P. shigelloides	3
			Serosanguinous exudate	20mm3	*A. hydrophila*	3
4	CID 07 014	8	Neck lesion	2+	*P. shigelloides*	3
			Muscle	4+	*P. shigelloides*	3
			Lungs	3+	*P. shigelloides*	3
			Kidney	2+	*P. shigelloides*	3
			Liver	4+	*P. shigelloides*	2
			Aorta	2+	*P. shigelloides*	3
			Umbilicus	2+	*P. shigelloides*	3

Case no	ID #	Positive samples	Sample origin	Cytology: leucocytes grade[#] / measure (mm^3)	Microorganisms	Colony growth score *
			Faeces	2+	*P. shigelloides*	3
5	CID 08 001	5	Neck lesion	4+	*P. shigelloides*	3
			Lungs	4+	*P. shigelloides*	2
			Kidney	4+	*P. shigelloides*	3
			Liver	4+	*P. shigelloides*	3
			Aorta	4+	*P. shigelloides*	3
6	CID 09 002	9	Neck muscle	2+	*A. hydrophila*	3
			Muscle	2+	*A. hydrophila*	3
			Lungs	2+	*A. hydrophila*	3
			Kidney	3+	*A. hydrophila*	3
			Liver	2+	*A. hydrophila*	3
			Spleen	4+	*A. hydrophila*	3
			Aorta	2+	*A. hydrophila*	3
			Blood	4+	*A. hydrophila*	3
			Serosanguinous exudate	0+	*A. hydrophila*	3

Case no	ID #	Positive samples	Sample origin	Cytology: leucocytes grade[#] / measure (mm^3)	Microorganisms	Colony growth score *
7	CID 09 008	9	Neck muscle	1+	*Citrobacter freundii*	3
					β-haemolytic Group C *Streptococcus* spp.	3
					Enterobacter aerogenes	3
			Muscle	1+	*Klebsiella pneumoniae*	3
					Escherichia coli	3
			Lungs	4+	Contaminated with mixed species	3
			Kidney	1+	*Citrobacter freundii*	3
					K. pneumoniae	3
			Liver	1+	*K. pneumoniae*	3
					E. coli	3
			Aorta	1+	Contaminated	3
			Serosanguinous exudate	0	Sterile	0
			Urine	14 mm3	*Enterobacter cloacae*	1
			Blow hole	0	Non-*albicans Candida* spp.	3

Case no	ID #	Positive samples	Sample origin	Cytology: leucocytes grade[#] / measure (mm^3)	Microorganisms	Colony growth score *
8	CID 10 002	11	Neck muscle	4+	*A. hydrophila*	3
					β-haemolytic Group C *Streptococcus* spp.	3
			Muscle	2+	*A. hydrophila*	3
					β-haemolytic Group C *Streptococcus* spp.	3
			Lungs	4+	*A. hydrophila*	3
					β-haemolytic Group C *Streptococcus* spp.	3
			Kidney	4+	*A. hydrophila*	3
					β-haemolytic Group C *Streptococcus* spp.	3
			Liver	4+	*A. hydrophila*	2
			Urine	23mm3	*A. hydrophila*	1
			Abscess	4+	*A. hydrophila*	3
					β-haemolytic Group C *Streptococcus* spp.	3
					P. shigelloides	3

Case no	ID #	Positive samples	Sample origin	Cytology: leucocytes grade[#] / measure (mm^3)	Microorganisms	Colony growth score *
			Uterus	2+	*A. hydrophila*	3
					β-haemolytic Group C *Streptococcus* spp.	3
			Amniotic fluid	4+	*A. hydrophila*	3
					β-haemolytic Group C *Streptococcus* spp.	2
			Blood	4+	*A. hydrophila*	3
					β-haemolytic Group C *Streptococcus* spp.	3
			Blow hole	2+	*A. hydrophila*	2
					β-haemolytic Group C *Streptococcus* spp.	3

*Colony growth score: 0 = Sterile, 1 = Present, 2 = Some, 3 = Numerous. [#]Cytology: 0 = Absent, 1+ =Rare, 2+ = Some, 3+ = Numerous, 4+ = Very numerous.

4.4 Discussion

The necropsies performed on the eight MRID revealed three critical observations indicative of disease. Firstly, calves were significantly more likely to have neck lesions and associated swelling than adults. Secondly, the dolphins displayed signs of pathology/disease that would be affecting their health status. For example, the five calves necropsied exhibited three types of disease pathology: gangrenous myonecrosis of the neck musculature; inflammation of the umbilical and genital region suggestive of infection and/or immunosuppression (Gulland, 2009); and hepatomegaly which has been linked to exposure to contaminants or pollutants in humans (Hsu et al., 2016; Lin et al., 2016; Lu et al., 2001; Serrano, 2014). In the three adults necropsied there was evidence of lung pathology indicating pneumonia, lymphadenomegaly indicative of inflammation, and coagulopathy with multiple species of bacteria detected in a range of tissues and organs. Both adults and calves were found to have skin lesions which may be indicative of endemic disease or immunosuppression at the population level, and as such this was examined further in Chapter 6 in live dolphins. Thirdly, *A. hydrophila* and *P. shigelloides* were each cultured from four dolphins. These species are believed to be commensals in dolphins (Buck et al., 2006; Pereira et al., 2008), and their role in the mortality of the dolphins investigated in the current study is unknown.

4.4.1 Incidental findings

This study is, to my knowledge, the first detailed investigation into mortalities of MRIDs and the pathology associated with these dolphins. Importantly, there were several incidental findings from this study.

4.4.1.1 Stomach contents

The lack of stomach contents, other than sand, in all but one (adult) case in this study is highly suggestive that these animals were not feeding prior to their death. These findings are in contrast to those of a study of 103 stranded bottlenose dolphins (*Tursiops truncatus*) in South Carolina Waters where 80% of the dolphins (immature/juveniles and mature adults) had stomach contents (Pate and McFee, 2012). In their study, they compared the stomach contents of dolphins with evidence of direct anthropogenic interaction (e.g. fishing entanglement or boat strikes) but were otherwise healthy, with those that had no evidence of anthropogenic interaction and appeared to die of natural causes. They found no difference between the two groups and suggested that the stomach contents of dolphins that died an acute or sudden death, such as from fisheries entanglement, would be a good indicator of the normal diet of the species. In contrast, they concluded that a dolphin that was diseased or compromised prior to stranding would have restricted ability to obtain food or prey and hence more likely to have an empty stomach (Pate and McFee, 2012). This was confirmed in a study by Yu and Xia (2013) in a female captive Irrawaddy dolphin that originated from the Mekong River, Vietnam that suffered anorexia for one day prior to her death, and was diagnosed with a bacterial infection of *Klebsiella* spp. and *Staphylococcus aureus*. The empty stomachs found in the current study are of concern and do not appear to be typical for dolphins, although further studies are required to investigate the stomach contents of MRID. In this study, six cases had small amounts of sand/sediment in their alimentary tracts. This may indicate that the dolphins had swallowed matter from the riverbed prior to their death, which would explain this ingested sediment (Parsons and Jefferson, 2000).

4.4.1.2 Skin lesions

Skin lesions were found in both adults and calves in the current study. Skin disease has been reported in many cetacean species across the globe (Hart et al., 2012, Van Bressem et al., 2003; Van Bressem et al., 2009; Van Bressem et al., 2014) and has previously been reported in *O. brevirostris* in Malaysia, India and Bangladesh (Van Bressem et al., 2014). Viruses have been implicated in skin lesions in dolphins with herpes virus linked to lesions in a study by Hart et al. (2012) and pox viruses identified in lesions in the studies by Fury and Reif (2012) and Van Bressem and Van Waerebeek (1996). Van Bressem et al. (2003) reported the presence of pox virus in tattoo-like lesions in all age categories, similar to the lesions observed in the current study. The findings of skin lesions in both adults and calves in the current study support a generalised cause of skin lesions within the population, similar to that suggested by Van Bressem et al. (2003). Although generally considered non-fatal, skin disease may be indicative of poor animal health or exposure to anthropogenic or environmental insults (Hart et al., 2012). Importantly Van Bressem et al. (2003) found that skin lesions may be suggestive of the presence of immune suppression and the results of a study investigating the prevalence of skin lesions in the MRID population are presented in Chapter 6 of this thesis.

In all three adults there were skin wounds suggesting aggressive interactions with conspecifics. Marley et al. (2013) found that tooth rake scars and skin "nicks" were often inflicted in dolphins during agonistic interactions with conspecifics. These can be used as an indirect indicator of conspecific aggression in the wild, where aggressive events may be difficult to observe (Marley et al., 2013).

Both Scott et al. (2005) and Marley et al. (2013) found that male dolphins exhibit more scars than females, largely due to increased aggression between males over access to females (Scott et al., 2005). Scott et al. (2005) also found that females in oestrus were significantly more likely to have tooth rakes than pregnant females or those with a dependent calf. However, in the current study the numbers were insufficient to confirm if sex played a role in the severity and frequency of skin lesions. These skin lesions could potentially serve as entry portals for opportunistic pathogens, resulting in disease. Future research is required to understand both the aetiology and consequences of these potentially aggressive interactions in MRIDs.

4.4.1.3 Hepatomegaly

The hepatomegaly observed in this study may be due to chronic exposure to pollutants and contaminants in the Mekong River. Unfortunately studies on dolphins with hepatomegaly have not previously been reported in the literature; although heavy metals have been associated with this condition in humans (Hsu et al., 2016; Lu et al., 2001; Serrano, 2014). Furthermore, heavy metals (Lin et al., 2016) and xenobiotic substances (Cave, 2020; Huang et al., 2005) have been shown to induce cellular damage in human liver cell lines. In two of the current cases fibrin deposits and hepatic lipidosis were detected. Fibrin is commonly induced in animal models (rat/mice) with acute and chronic liver disease or injury (Kopec and Luyendyk, 2016). Kuiken (2010, pers. comm.) interpreted the histological findings of hepatic lipidosis and atrophy as indicating the presence of two processes: firstly chronic emaciation, leading to atrophy of the hepatocytes; and secondly mobilisation of lipids from adipose tissue and their subsequent accumulation in hepatocytes due to a lack of nutrients required to synthesise and export low density lipoproteins. Research into hepatomegaly in relation to contaminants is explored further in the toxicological study outlined in Chapter 7.

4.4.1.4 Dental malformations

Dental malformations were found in two of the three adult cases. This finding has not been reported previously in any *Orcaella* species. These malformations may be used as "dental weaponry" against conspecifics, as has been reported in 18 other odontocete species, including Risso's dolphins (*Grampus griseus*), narwhal whales (*Monodon monoceros*), sperm whales (*Physeter catodon*) and members of the family Ziphiidae (MacLeod, 1998). Further research is needed to understand the causes and consequences of these dental abnormalities, and the prevalence in the general population of MRID.

4.4.1.5 Twins in *Orcaella brevirostris*

In this study, twin foetuses were found in Case 8. To the author's knowledge this is the first report of twins in *Orcaella* spp. Twins in dolphins are infrequently reported, with records in the literature in only four dolphin species, namely a striped dolphin (*S. coeruleoalba*) (Tobayama et al., 1970), bottlenose dolphins (*T. truncatus*) (Dabin et al., 2004; Kompanje, 2005; Aytemiz et al., 2014; Gray & Conklin, 1974; LeCave, 1991), a short-beaked common dolphin (*Delphinus delphis*) (González et al., 1999), and an Atlantic white-sided dolphin (*Lagenorhynchus acutus*) (Davison et al. 2016). There are no studies in the literature documenting maternal mortality with twins in dolphins. However, Yu and Xia (2013) documented maternal mortality from chorioamnionitis associated with *Klebsiella* spp. and *Staphylococcus aureus* infection in a captive gravid *O. brevirostris,* with a singleton pregnancy, that was captured from the Mekong River Delta in Vietnam 14 months previously in 2008. Physiological changes associated with pregnancy can include immunosuppression and exacerbation of underlying disease states (Kaaja and Greer, 2005). However; these

impacts could potentially be even greater in twin pregnancies as Santana et al. (2016) reported, that in humans twin pregnancies are associated with greater severe maternal morbidity and a higher rate of maternal death than in singleton pregnancies. Similarly, in developing nations, twin pregnancies in humans are a significant risk factor for maternal mortality (Vogel et al., 2013). Thus, it is possible that the twin pregnancy detected in Case 8 contributed to her mortality.

4.4.1.6 Parasites

Bradyzoites of a *Sarcocystis* spp. were detected in the skeletal muscle of one adult (Case 7) in this study. This is the first report of *Sarcocystis* spp. bradyzoites in *O. brevirostris*, although they have been reported in several stranded dolphins, including in 39% (n=28) Atlantic white-sided dolphins (*Lagenorhynchus acutus*) (Ewing et al., 2002) and a striped dolphin (*S. coeruleoalba*) (Resendes et al., 2002). However, there is a paucity of literature about the prevalence, distribution and lifecycle of *Sarcocystis* spp. in dolphins. It is assumed that the dolphin acts as the definitive host (carnivore) in the parasite's life cycle; however this requires further investigation in this population to identify the potential effects, if any, of the protozoa on the health of the dolphins.

4.4.1.7 Potential infanticide

The final incidental finding in this study was that of possible infanticide in one calf (Case 1). This calf had a fractured rib, which, given the lack of evidence for trauma from a boat strike or other anthropogenic event, may be indicative of trauma from another member of the population. Infanticide has been documented in several cetacean populations, for example in bottlenose dolphins (*Tursiops* spp.) (Kaplan et al., 2009; Patterson et al., 1998), Indo-Pacific

humpback dolphins (*S. chinensis*) (Zheng et al., 2016), and Amazon River dolphins (*Inia geoffrensis*) (Bowler et al., 2018), however it is considered a rare event (Wells, 2014) and remains poorly understood (Zheng et al., 2016). Infanticidal behaviour of the MRIDs has been witnessed on several occasions (Limsong et al., 2017), however due to a lack of evidence of blunt trauma on gross necropsies, it is not believed to be the principal aetiology of calf mortality.

4.4.2 Implications of potential disease signs in the Mekong River Irrawaddy dolphins

It has previously been argued that neck lesions in dolphins could be post mortem artefacts and are not a pathological condition (CSG, 2012a; Gulland, 2014; Smith, 2017). However, several observations from the current study question this hypothesis. Firstly, in the five MRID calf cases with neck lesions, no evidence of blood pooling was detected in the cervical region and gross changes were observed both superficially and deeper in the musculature. This is in contrast to post mortem hypostasis (lividity or *livor mortis*) when blood settles in the blood vessels under the influence of gravity after death (Pollanen et al., 2009). Lividity was apparent in one adult (Case 8), however this dolphin did not have a neck lesion. Secondly, regardless of the positioning of the dolphin carcases in this study, the bruising remained in the same location. In a study by Vanezis (2001) it was reported that by moving the cadaver into a different position pooled blood would drain to a secondary position in post mortem hypostasis, whereas true bruising would remain in the same position. Finally, the neck lesions in the cases reported here were wet or gas gangrene type-lesions. Gangrenous lesions located specifically in the neck region are not commonly reported in the literature for any species, although some cases in humans have been reported as cervical necrotising fasciitis (Vaid et al., 2002). Initially it was thought that *Clostridium* spp. could be involved in

the lesions observed in the current study, however clostridia were not cultured from any of the lesions. A range of aerobic and anaerobic non-clostridial bacterial species can cause gas gangrene, including some that were isolated in this study including: *Streptococcus* spp.; *A. hydrophila*; *K. pneumoniae*; *E. coli*; *M. morganii*; *Enterobacter* spp.; and *Citrobacter* spp. (Fatimi et al., 2007; Ghosh et al., 2009; Jayanth et al., 2016; Ohi et al., 1993). In addition, non-clostridial gas gangrene has been associated with trauma and acute pharyngolaryngeal inflammation in humans (Ohi et al., 1993), both of which were demonstrated in this study.

Necrotising fasciitis would appear the best description of the lesions seen in the five MRID calves, for several reasons. Firstly, in humans, it is frequently fatal (Cui et al., 2007; Diab et al., 2020; Shindo et al., 1997; Thomas and Meyer, 2012); secondly, the organisms isolated in this study have been cultured from necrotising fasciitis lesions in humans (Apisarnthanarak et al., 2008; Arroyo et al., 2010; Markov et al., 2007; Minnaganti et al., 2000; Monaghan et al., 2008); thirdly, it can affect human neonates (Pandey et al., 2008; Sakata et al., 2009; Zuloaga-Salcedo et al., 2005); and finally, infection can originate deep in the soft tissues, without an external portal of entry, often at sites of non-penetrating trauma such as a bruise (Stevens and Bryant, 2017). Necrotising fasciitis will be examined and discussed in more detail in the following chapter.

4.4.3 Implications for future research and policy

This study has identified a number of directions for future research into mortalities as outlined earlier. Additionally, further research investigating the microbiology of the neck lesions is required, as well as examining the immune status of the MRIDs in order to better understand the host/pathogen relationship and their role in disease causation in this

population. For example, the two pathogens, *A. hydrophila* and *P. shigelloides*, were identified in cases with neck lesions, lung pathology and other abnormalities in this study. While these bacteria are often considered commensals (Buck et al., 2006), they were found in tissues with clear evidence of pathological changes. For example, *A. hydrophila* was cultured from the amniotic fluid of the case with chorioamnionitis (Case 8), and also from this animal's urine in association with cystitis. Both *P. shigelloides* and *A. hydrophila* were also cultured from the pyogranulomatous exudate in the splenic abscess of this case. In addition, *A. hydrophila* was cultured in lesions associated with bronchopneumonia in Case 3. In this case Kuiken (2010 pers. comm.) found that the lymph nodes draining the lungs, showed no evidence of lymphoid hyperplasia which would be expected in cases of bronchopneumonia, which, together with the presence of hypocellularity of the splenic white pulp, suggests an ineffective immune response of this dolphin. This case demonstrates potential immunosuppression of the dolphin (host) and opportunistic infection from the pathogen (agent) (Kuiken, 2010 pers. comm.).

Based on the findings in this study the microbiology and associated neck lesions are further explored in Chapter 5. There is also a need to ascertain the aetiology of the hepatomegaly seen in this study which requires further histological examination of liver tissue. Additionally, the role of pollutants in the Mekong River needs investigating and this topic is examined further in Chapter 7. There is also an ongoing need to perform additional necropsies to build on the observations from the current study of a small number of necropsies. This would help increase the power of the data analyses. Finally, there is a need to quantify the extent of skin lesions in the MRID population. The prevalence of skin lesions in the MRID is further examined in Chapter 6 of this thesis.

4.4.4 Study limitations

There were several important limitations to the current study.

Firstly, it was not possible to do a complete histopathological analysis from the results obtained. This was due to rapid decomposition and autolysis of dead dolphins which, together with post mortem bacterial overgrowth, made it impossible to gain meaningful results from all samples, tissues and organs. This was largely attributable to the hot, humid environmental conditions at the time of death. Temperatures in the Mekong River region at the time when this study was conducted average 32˚C (Irvine et al., 2011). Thus, even in the two cases where the necropsy was commenced within two hours of death, rapid autolysis occurred. Similar observations were also reported in the study of Klein et al. (2016).

Secondly, Government bodies (MAFF and the DC) denied permission on several occasions to allow necropsies to be performed soon after the animal's death, resulting in carcasses being frozen for subsequent necropsy. The time to freezing and duration of freezing may have negatively affected both the bacterial viability and the histological structure of tissues through formation of ice crystal artefacts (Sen and Sharma, 2004; Chatterjee, 2014) making it harder to detect evidence of pathology and/or disease.

Thirdly, there was a delay from the time of sample collection for histology (2007-2009) and eventual analysis and processing in 2010. This arose from logistical constraints of getting samples out of the country with the necessary CITES permits, and obtaining permission from the Government of Cambodia. Additionally, the samples were not permitted to be shipped in formalin, so the samples were drained of formalin and re-fixed on arrival in the Netherlands. The process of formalin fixation of samples induces chemical changes and degradation in tissue DNA, RNA and protein, which can render subsequent interpretation unreliable

(Gnanapragasam, 2010; Chatterjee, 2014), particularly in samples that may be over-fixed or stored for several years prior to processing (Ammerlaan et al., 2018; Daugaard et al., 2015; Xie et al., 2011).

Fourthly, it was not possible to perform viral cultures due to a lack of -80˚C freezers or laboratory facilities for viral isolation/detection in Cambodia from 2007-2010. Future research is required to investigate the possible role of viruses in disease and mortalities in MRID.

Finally, because of the restriction to a CS2 state, the number of dolphins included in the study was very small. This small number limits statistical power of the analyses (Faber and Fonseca, 2014).

4.5 Conclusions

In this chapter the lesions and pathological findings from eight MRID are described and their potential role in mortality discussed. In particular there was a difference in the presence of neck lesions between adults and calves, with all five calves necropsied affected. As the MRID population is on the verge of extirpation, the findings of these necropsies provide some insight into the potential underlying aetiologies for the mortalities in this population. Whilst each case was unique, there were some similarities between them, with several cases demonstrating a component of immunosuppression and presence of potentially opportunistic bacteria/pathogens. Due to the finding of neck lesions in all of the five calves necropsied, further research examining these lesions and their associated microbiological fauna needs to be undertaken, so as to better understand the epidemiology of this disease process. This is examined further in the following chapter.

CHAPTER FIVE

Is necrotising fasciitis the cause of neck lesions in dead Mekong River Irrawaddy dolphins from Cambodia? A retrospective study

5.1 Introduction

In the previous two chapters neck lesions were identified as being more common in dead dolphin calves than in adults or juveniles. Currently, however, there is a paucity of knowledge relating to the aetiology of neck lesions in dolphins. In a detailed literature review of neck lesions with gas formation, necrotising (necrotizing) fasciitis was a prominent finding reported in humans and several other animal species, including dogs (*Canis lupus familiaris*) (Kulendra and Corr, 2008), cats (*Felis catus*) (Brachelente et al., 2007; Nolff and Meyer-Lindenberg, 2015; Plavec et al., 2016), neonatal crocodiles (*Crocodylus porosus*) (Bishop et al., 2007), a gorilla (*Gorilla gorilla*) (Allender et al., 2009) and a bottlenose dolphin (*Tursiops truncatus*) (Zappulli et al., 2005). Consequently, I decided to initially review the literature on necrotising fasciitis, and then examine the historical necropsy cases in MRIDs that had neck lesions, to determine if this disease could be playing a role in the pathogenesis of the neck lesions seen in the MRIDs.

5.1.1 Necrotising fasciitis overview

Necrotising fasciitis has a long history in humans, being first reported by Hippocrates in the 5th Century BC as a fatal infection which produced gross discolouration, swelling and eventual gangrene (Morantes and Lipsky, 1995). This condition has been also referred to as "*hospital gangrene*" in 1871 during the American civil war, (Bain, 1999; Dale et al., 1999),

and "*haemolytic streptococcus gangrene*" by Meleney in 1924. Since then it has also been referred to as, "*Meleney ulcer", "malignant ulcer", "gangrenous ulcer", "putrid ulcer", "Meleney's gangrene*", "*phagedena*", "*phagedena gangrenosum", "acute dermal gangrene", "suppurative fasciitis", "necrotizing myositis", "synergistic necrotizing cellulitis", "necrotizing erysipelas", "sanguineous erysipelas", "gangrene", "symbiotic gangrene", "progressive post-operative bacterial gangrene", "acute infective gangrene", "Fournier's gangrene", "suppurative fasciitis progressive gangrenous infection of the skin"* and *"spreading gangrenous inflammation"* (Bain, 1999; Dale et al., 1999; Maynor and Kulkarni, 2011; Meleney, 1924; Shindo et al., 1997; Wong et al., 2003). The term "*necrotising fasciitis"* was first used by Wilson (1952) and Wong et al. (2003) believed that this term was the most accurate description based on the key presenting features in humans of this infectious process.

Necrotising fasciitis describes a potentially fatal, rapidly progressive, disseminating, soft-tissue bacterial infection located in the deep fascia, causing secondary necrosis of the subcutaneous tissues and the superficial and deep fascial layers, often containing gas-forming organisms resulting in the presence of subcutaneous gas (Dale et al., 1999; Krebs et al., 2001; Maynor and Kulkarni, 2011; Shindo et al., 1997; Stevens, 1995). It is characterised by extensive necrosis of the superficial fascia, rapidly spreading to involve the surrounding tissues with associated systemic toxicity and shock (Shindo et al., 1997), which can result in multiple organ system failures (Bain, 1999; Dale et al., 1999).

5.1.2 Epidemiology of necrotising fasciitis

Necrotising fasciitis in humans is often associated with comorbidities, such as immunosuppression (Bain 1999, Diab et al., 2020; Trent and Kirsner, 2002), or disease states, such as liver disease or diabetes (Dale et al., 1999; Shindo et al., 1997; Wong et al., 2003), that reduce the host's defence mechanisms (Ozturk et al., 2005). For example, Ibekwe et al. (2011) demonstrated a relationship between the immunosuppressive effects of viruses and bacterial colonisation, resulting in necrotising fasciitis. In addition to comorbidities, cases of necrotising fasciitis in humans have also been documented in young, apparently healthy, individuals with no history of underlying disease, having only sustained minor blunt trauma resulting in haematoma, deep bruising or muscle strain, with several of these cases displaying clinical signs within 24-72 hours of the traumatic event (Dale et al., 1999; Shindo et al., 1997; Stevens, 1995). The causal web of necrotising fasciitis is depicted in Figure 5-1.

Based on the pathways depicted in Figure 5-1, the most likely causal pathways for MRID mortality would be via immunosuppression, resulting in disease or necrotising fasciitis. Factors that can lead to immunosuppression include age (young or old), poor nutritional state, stress, pathogenic infection, environmental contaminants, liver pathology, and poor water quality. In the MRID calves, the most likely pathway for necrotising fasciitis would be via opportunistic pathogen infection, specifically commensals, as a result of skin trauma such as bite wounds and tooth rakes from other dolphins, as was evident in the necropsy examinations in Chapter 4. Skin trauma can result in necrotising fasciitis directly or indirectly if the calves are immunosuppressed. In Chapter 4, hepatomegaly was also a consistent feature, which may also facilitate the development of necrotising fasciitis.

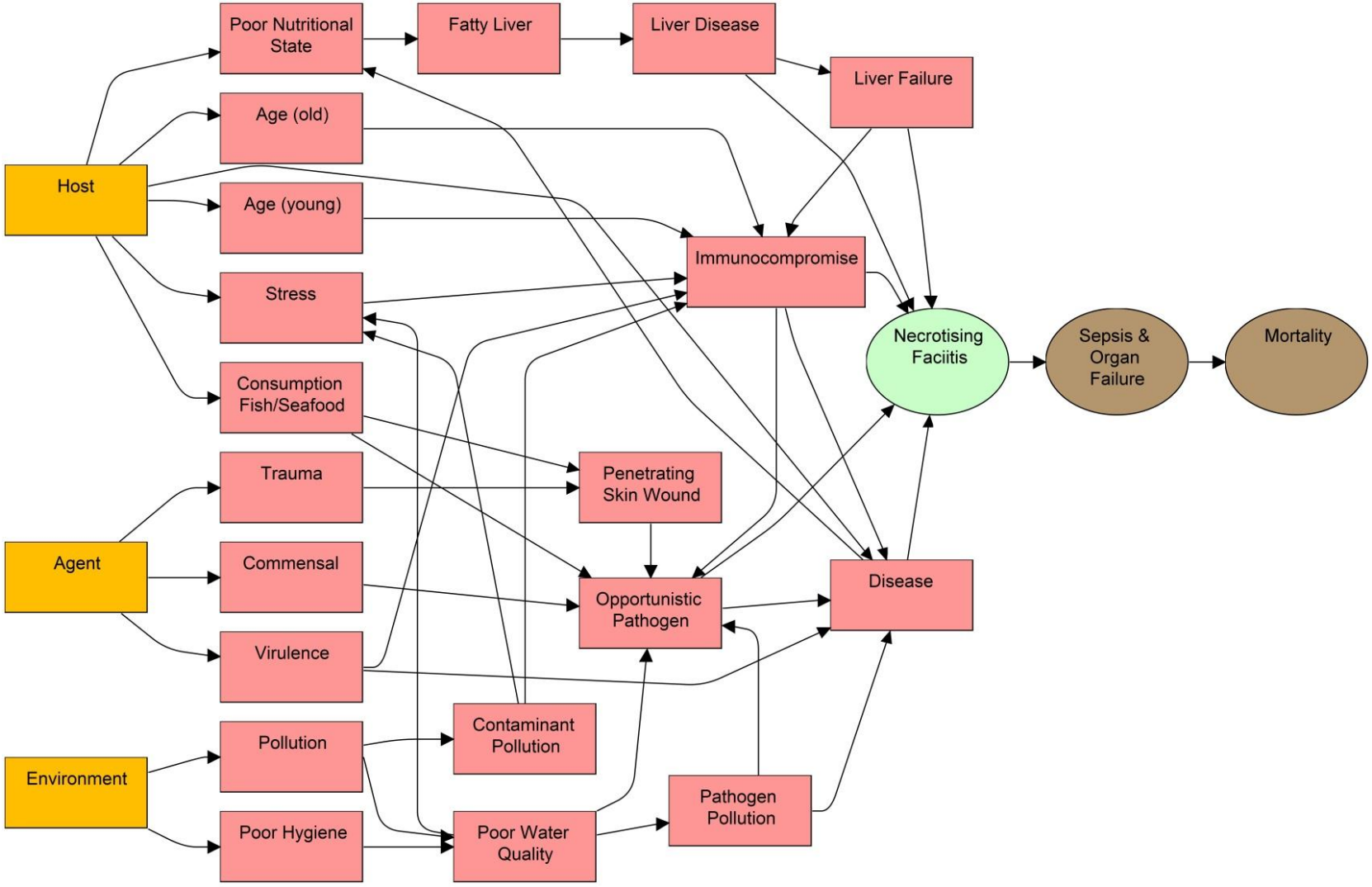


Figure 5-1: Causal web of necrotising fasciitis epidemiology.

5.1.2.1 Causative organisms

The bacteria cultured from lesions of necrotising fasciitis have included: *E. coli*; *M. morganii*; *Klebsiella* spp. (Dale et al., 1999; Gangopadhyay et al., 2008; Krebs et al., 2001; Wong et al., 2003); *Staphylococcus* spp. (Dale et al., 1999); ß-haemolytic *Streptococcus* spp. (Dale et al., 1999; Ozturk et al., 2005; Shindo et al., 1997; Wong et al., 2003; Zappulli et al., 2005); *Enterococcus* spp.; *A. hydrophila* (Apisarnthanarak et al., 2008; Arroyo et al., 2010; Markov et al., 2007; Minnaganti et al., 2000; Monaghan et al., 2008; Wong et al., 2003); *Peptostreptococcus* spp.; and *Fusobacterium* spp. (Dale et al., 1999), along with yeasts (Shindo et al., 1997). The wide range of causative organisms is, in part, correlated with their presence as normal flora in the body tissues/region adjacent to the affected site (Brook and Frazier, 1995). In addition, certain pre-disposing factors may be correlated with the presence of particular bacterial species, for example: *Clostridium* spp. with trauma; and species belonging to the family *Enterobacteriaceae* with immunosuppression (Brook and Frazier, 1995).

5.1.2.2 Pathophysiology

Necrotising fasciitis can be difficult to recognise in the early stages of the disease (Maynor and Kulkarni, 2011), as skin manifestations lag behind the disease progression occurring under the skin (Wong et al., 2003). As such, the disease may take a week to become fulminant, with the onset of clinical signs progressing gradually over three to four days (Brook and Frazier, 1995). In addition, polymicrobial infections tend to have a more indolent onset, which may delay early detection (Brook and Frazier, 1995). However, this indolent onset can be followed by a rapid progression and decline in the host within a few hours to a

day (Brook and Frazier, 1995; Maynor and Kulkarni, 2011). This is because a state of extreme metabolic catabolism develops (Dale et al., 1999), causing the infection to spread rapidly, resulting in overwhelming sepsis followed by multi-organ system failure as a result of toxic shock, and subsequently death (Dale et al., 1999; Shindo et al., 1997).

5.1.2.3 Diagnosis of necrotising fasciitis

A diagnosis of necrotising fasciitis in humans is predominantly clinical, with a high index of suspicion (Brook and Frazier, 1995; Diab et al., 2020; Thomas and Meyers, 2012), as described in studies by Brook and Frazier (1995) and Stevens (1995), however the presence of bullae filled with serous fluid should raise suspicion of the condition, and may be an important diagnostic clue (Wong et al., 2003).

Cervical necrotising fasciitis is challenging to diagnose (Thomas and Meyer, 2012). For example, routine laboratory tests are largely nonspecific, as there may or may not be a leucocytosis, and blood culture may or may not be positive (Dale et al., 1999). Thomas and Meyer (2012) conducted a study to evaluate the utility of two laboratory-based diagnostic tests for distinguishing necrotising fasciitis from non-necrotising infection when specifically applied to neck infections (Table 5-1). Their study also confirmed the difficulty in definitively diagnosing necrotising fasciitis using laboratory tests, particularly when it affected the neck region. Goh et al. (2014), in a systematic review of the literature, found that 81% of patients with necrotising fasciitis (n=1463) had localised swelling, which may greatly increase clinical suspicion, and thus prove more diagnostic than laboratory tests alone.

Table 5-1: Laboratory tests[†] and test parameters to diagnose cervical necrotising fasciitis

Laboratory test	Reference range/value	Sensitivity % (95%CI)	Specificity % (95%CI)
WBC[*] and Na+[*]	>15.4 x 10^3/μL <135 mEq/L	24 (7.8-50.2)	81 (68.8-88.9)
LRINEC[#]	score ≥ 6	56 (22.7-84.7)	60 (27.4-86.3)

[†]Adapted from Thomas and Meyer (2012)
[*] White blood cell count (WBC) and serum sodium (Na+) levels
[#] Laboratory Risk Indicator for Necrotising Fasciitis (LRINEC)

Despite the difficulty in diagnosis through laboratory testing, it is still recommended that tissue and fluid from the affected site be cultured and any bacteria isolated assessed for antibiotic sensitivity testing (Dale et al., 1999). A positive Gram stain from infected tissue and/or blood culture can further aid in the diagnosis of the condition (Trent and Kirsner, 2002).

In necropsy examinations, definitive diagnosis of necrotising fasciitis is made through pathological examination of the affected tissue (Ozturk et al., 2005). A characteristic gross finding is the ability to dissect between fascia and subcutaneous planes with little resistance, with thin murky fluid, dark-brown coloured fascia, and patchy greenish liquefaction necrosis usually present (Shindo et al., 1997).

The aim of the study outlined in this chapter was to evaluate 16 post mortem cases of MRID that had microbiology results, and determine the likelihood that necrotising fasciitis was responsible for the neck lesions present in 13 of these dolphins.

5.2 Materials and methods

5.2.1 Necropsy protocol

Retrospective data on necropsies that were carried out between March 2007 and March 2010 were used in this study. I performed complete necropsies on 15 of the 16 selected cases. One case (Case 13) I was permitted to take photos and collect samples from the neck region, however the Cambodian government conducted the complete internal necropsy, of which no information was made available. Of the 16 cases, 13 had neck lesions.

The materials and methods for the 15 post-mortem examinations I conducted were as previously described in Chapter 4. Seven of the eight cases from Chapter 4 (Cases 2-8) were included in this study (Cases 2, 4, 5, 6, 11, 14 and 16 in the current study). Case 1 from Chapter 4 was excluded as it did not have microbiology testing.

The blubber layer of each dolphin was measured in 12 locations of the body (Figure 5-2) using vernier calipers and the average calculated. The BMI for each animal was calculated as the weight (kg) divided by the length $(m)^2$, and compared between age classes.

The commonly encountered pathological findings for the dolphins with neck lesions were recorded, and compared to the three dolphins without neck lesions.

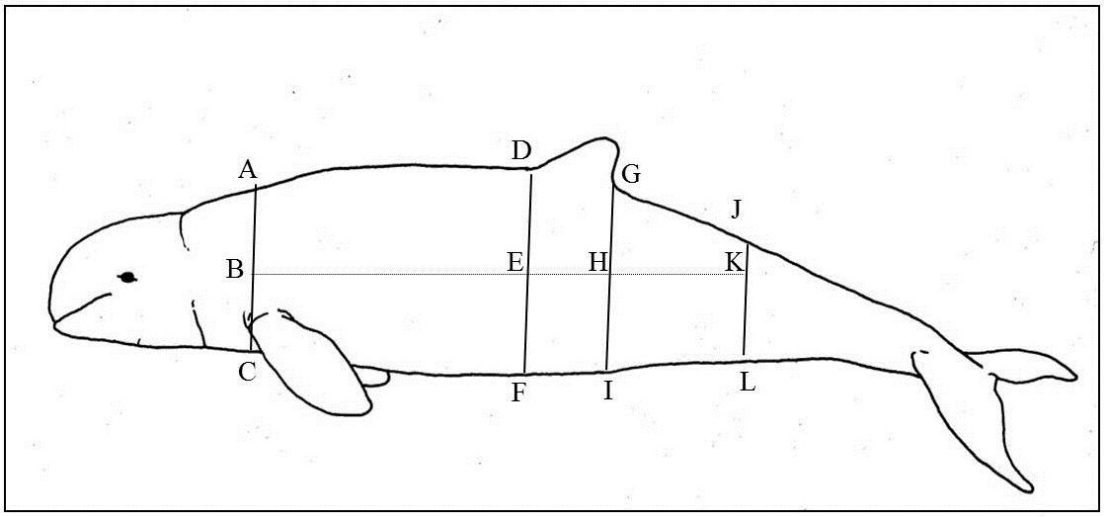


Figure 5-2: Blubber measurement locations A-L, taken in mm.

"Neck lesions" (NL's) in this study were characterised by the following criteria:

1. The location(s), as outlined in Figure 5-3
2. The nature of the lesion, defined as swelling, and/or a red to dark purple-black area of discolouration visible on the dermis or epidermis, occurring with dark liquefactive necrosis and musculature discolouration on cut section(s). In addition, on gross examination, the neck musculature must have an obvious line of demarcation that was distinct from other "non-affected" and presumably normal muscles of the body.

The neck lesions varied in size and extent, and, as such, they were characterised accordingly (Figure 5-3) and assigned a grade depending on the tissue involvement.

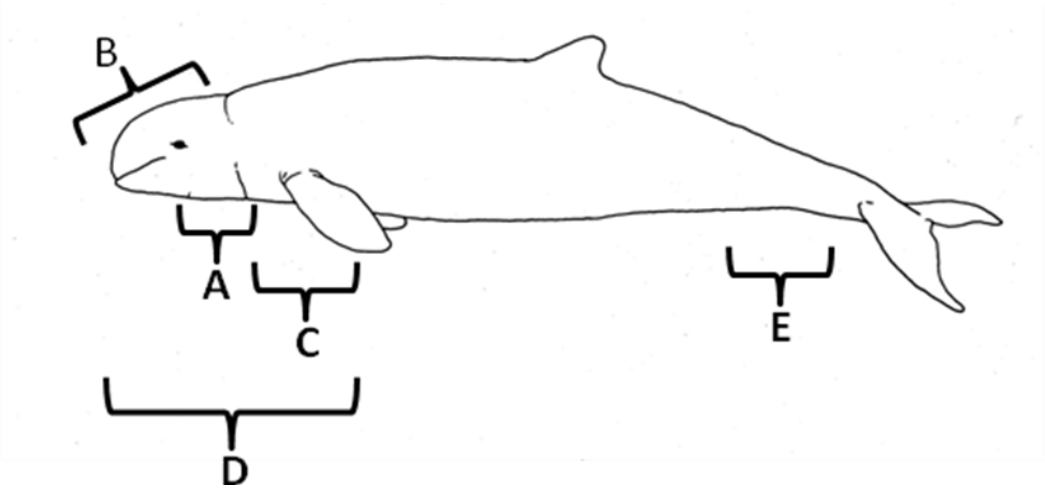
Category for describing neck lesions	Description
	
Grade 1	Neck lesion on the ventral and lateral aspect of the cervical region (A).
Grade 2	Extension of the neck lesion cranially and dorsally from the cervical region (B).
Grade 3	Extension of the neck lesion caudally, including the upper pectoral muscles, and flipper insertions (C).
Grade 4	Neck lesion extends including A, B and C (D).
Grade 5	Neck lesion (A ± B/C) present in association with discoloration of the peduncle (tail stock) caudal to the umbilicus (E).

Figure 5-3: System for characterising the extent of the neck lesion identified and the association with similar lesions on the tail stock.

5.2.1.1 Microbiological and cytological sample collection

The materials and methods for microbiological sample collection and cytological analysis were as previously described in Chapter 4. Thirteen cases with neck lesions had neck lesion muscle collected aseptically, and nine of these had corresponding normal appearing muscle adjacent to the lesion collected aseptically for comparison. Adjacent muscle was collected from the neck region in all cases, except for Case 4, 5, 8 and 9 where the adjacent muscle was

collected from the ventral thoracic region. In all three cases without neck lesions, normal appearing muscle was collected from the neck. Case 13 had two small neck muscle samples collected due to restrictions by the Cambodian Government, Case 11 had duplicate samples of neck muscle collected, and Case 14 had triplicate neck muscle samples collected. All samples were included in the analysis.

Urine was collected aseptically via cystocentesis when present (Cases 9, 14 and 16).

5.2.1.1.1 Microbiological data analysis

Data were summarised according to the microorganisms detected in all 16 cases. The species/types of microorganisms detected were then compared between samples from the 13 dolphins with neck lesions to the three dolphins without a neck lesion. Finally, the data were compared from samples within individual animals (samples of areas with lesions vs. samples from apparently normal tissue).

5.2.1.1.2 Cytological data analysis

The data from cytology were compared between dolphins with neck lesions (neck lesion muscle) to those dolphins without neck lesions (grossly normal neck muscle). In the dolphins with neck lesions, samples of the affected tissue were also compared with adjacent normal appearing muscle in these dolphins. Cytological samples from the liver of dolphins with neck lesions were also compared to those without neck lesions. These analyses were then repeated comparing neck lesion samples to adjacent normal muscle within individual dolphins (paired data). Where duplicated and triplicated muscle samples were collected, the median of these

samples were used in the analysis to reduce bias and overestimation of sampled population size.

5.2.1.2 Histology

The sample collection, storage and analysis for histological analysis by Prof. T. Kuiken were as previously described in Chapter 4. Samples from nine carcasses were prioritised according to their carcass score (CS2 (n=7) and CS 3 (n=2)) and submitted for histological analysis.

5.2.1.3 Statistical analyses

Bacterial species, pathological abnormalities and cytology were all compared in neck lesion samples and in normal muscle samples.

Statistical analyses were as described previously in Chapter 3 for 2x2 contingency tables using the Pearson's Chi-square test or Fishers' exact tests; and prevalence and 95%CI and odds ratios (OR) and 95%CI calculated using the exact binomial method. Normality of the data was checked using a Shapiro-Wilk Test. For non-parametric cytological data a Mann-Whitney U test was used with a ties correction, and Yates' correction for continuity due to the small sample set, the normal approximation p-value was given in this study. Paired data from individual dolphins were analysed using a Wilcoxon Signed-Rank test. All data were analysed using the Real Statistics Resource Pack (Release 5.4) for Excel (2010) (Zaiontz, 2018).

5.3 Results

5.3.1 Necropsy results

A summary of the 16 necropsied cases is presented in Table 5-2. This study included five adults, three juveniles and eight calves, of which 10 were female and six were male.

The BMI in the cases ranged from 8.0-13.5 ($\bar{X}$ = 12.1, SD 2.3) in calves; 10.4-22.4 ($\bar{X}$ = 17.2, SD 6.14) in juveniles and 13.5-26.1 ($\bar{X}$ = 21.4, SD 5.2) in adults. There was no significant difference in the BMI between adults with and without neck lesions (p=0.76) and similarly for juveniles (p=0.22). The influence of neck lesions on the BMI of calves could not be assessed as no calves without neck lesions were necropsied.

5.3.1.1 Extent of neck lesions

Of the 13 dolphins with lesions, all had lesion(s) at location A of the neck (100%; 95%CI 75.3, 100). Of these 13 cases, 23.1% (95%CI 5.0, 53.8; n=3) were classified as Grade 1; 7.7% (95%CI 0.2, 36; n=1) as Grade 2; 15.4% (95%CI 1.9, 45.4; n=2) as Grade 3; 15.4% (95%CI 1.9, 45.4; n=2) as Grade 4; and 38.4% (95%CI 13.0, 68.4; n=5) as Grade 5 (Table 5-3).

The results of the pathological abnormalities and gross post mortem findings are listed in Table 5-4 and are summarised in Table 5-5.

Table 5-2: Necropsied dolphins with tissue samples submitted for microbiological assessment from 2007-2010

Year	Case #	ID #	Specimen number	Age	Sex	CS	Neck lesion	Blubber thickness (mm)	BMI (kg/m^2)	Histology
2007	1	78	CID 07 007	Calf	Female	3	+	17.8	13.54	×
	2	80	CID 07 009	Calf	Male	2	+	17	10.41	✓
	3	82	CID 07 011	Adult	Female	3	+	38.3	26.09	×
	4	83	CID 07 012	Calf	Female	2	+	13.8	14.85	✓
	5	85	CID 07 014	Calf	Female	2	+	9.8	7.98	✓
2008	6	88	CID 08 001	Calf	Female	2	+	15.5	13.41	✓
	7	86	CID 08 002	Calf	Male	3	+	12.4	12.74	×
	8	89	CID 08 003	Juvenile	Female	3	+	15.1	18.81	×
	9	90	CID 08 005	Adult	Male	2	+	30	21.90	✓
2009	10	94	CID 09 001	Juvenile	Female	3	+	15.9	22.38	✓
	11	92	CID 09 002	Adult	Female	2	-	17.8	13.54	✓
	12	95	CID 09 003	Calf	Male	3	+	13.3	13.41	✓
	13*	98	CID 09 006	Juvenile	Female	2	-	17	10.41	×
	14	96	CID 09 008	Adult	Male	2	-	38.3	26.09	✓
2010	15	101	CID 10 001	Calf	Female	2	+	20.9	10.17	×
	16	102	CID 10 002	Adult	Male	3	+	31.9	19.28	×

*Necropsy conducted by the Cambodian Government; ID # Identification number; + Present; - Absent; ✓ Procedure conducted; × Procedure not conducted; CS = Carcass Condition Score; BMI: Body Mass Index

Table 5-3: Grading and location of lesions in 13 cases that had neck lesions

		Region of the carcass with lesions					
Grade	**Case #**	**A Neck**	**B Ventral head**	**C Thorax**	**D Head and thorax**	**E Peduncle**	**Total % (95%CI)**
1	12	+	-	-	-	-	
	15	+	-	-	-	-	23.1 (5.0, 53.8)
	16	+	-	-	-	-	
2	3	+	+	-	-	-	7.7 (0.2, 36.0)
3	5	+	-	+	-	-	15.4 (1.9, 45.4)
	8	+	-	+	-	-	
4	1	+	-	-	+	-	15.4 (1.9, 45.4)
	9	+	-	-	+	-	
5	2	+	-	-	-	+	
	4	+	+	-	-	+	
	6	+	-	-	-	+	38.4 (13.9, 68.4)
	7	+	-	-	-	+	
	10	+	+	-	-	+	
Total % (95%CI)		100 (74.9,100)	23.1 (5, 53.8)	15.4 (1.9, 45.4)	15.4 (1.9, 45.4)	38.4 (13.9, 68.4)	

+: Present; - Absent

Table 5-4: Gross pathological findings from 16 necropsied dolphins

	Case #	Hepato-megaly	Serosanguinous		Haemo-thorax	Swelling		Tattoo lesions	Other skin lesion	Petechial haemorrhages	Stomach contents
			Exudate	Orifices		U	G				
Neck lesion(s) present	1	-	-	-	-	-	+	-	+	-	-
	2	+	+	BH, O	-	-	-	+	+	-	-
	3	+	+	BH, O, U	+	-	-	-	-	-	-
	4	+	-	-	+	+	+	-	+	+	-
	5	+	+	O, U, E	-	+	+	-	+	-	-
	6	+	+	O	-	+	+	-	+	+	-
	7	+	-	-	-	-	+	-	+	-	-
	8	+	+	O	+	-	+	-	+	-	-
	9	+	+	U, G	+	-		-	+	+	+
	10	-	-	-	+	+	+	-	+	+	-
	12	+	-	-	-	-	-	+	+	-	-
	15	-	-	-	-	-	-	-	+	-	?
	16	+	+	O	-	-	-	+	+	-	-

	Case #	Hepato-megaly	Serosanguinous		Haemo-thorax	Swelling		Tattoo lesions	Other skin lesion	Petechial haemorrhages	Stomach contents
			Exudate	Orifices		U	G				
No neck lesions present	11	-	-	-	-	-	+	-	+	-	-
	13	?	+	BH, O	?	-	-	+	+	-	?
	14	-	+	BH, O, U	+	-	-	-	-	-	+

+ Present; - Absent; ? Unknown; BH = Blow Hole; O = Oral; U = Umbilical; E = eye; G = Genital

Table 5-5: Summary of major external observations (n=16) and post mortem findings (n=15) in dolphins with and without neck lesions

External examination (n=16)	**Finding**		**Neck lesions present n=13**	**Neck lesions absent n=3**
	Blood pooling orifices	n % (95%CI)	7 53.85 (25.1, 80.8)	0 0 (0, 70.8)
	Genital swelling	n % (95%CI)	7 53.85 (25.1, 80.8)	1 33.33 (0.8, 90.6)
	Umbilical swelling	n % (95%CI)	4 30.77 (9.1, 61.4)	0 0 (0, 70.8)
	Tattoo skin lesions	n % (95%CI)	3 23.08 (5.0, 53.8)	1 33.33 (0.8, 90.6)
	Trauma skin lesions	n % (95%CI)	12 92.31 (64.0, 99.8)	3 100 (29.2, 100)
Internal post mortem examination (n=15)	**Finding**		**Neck lesions present n=13**	**Neck lesions absent n=2**
	Hepatomegaly	n % (95%CI)	10 76.92 (46.2, 95)	0 0 (0, 84.2)
	Haemothorax	n % (95%CI)	5 38.46 (13.9, 68.4)	1 50 (1.3, 98.7)

n=number of individual cases

Overall there was no significant difference in the presence of hepatomegaly in dolphins with neck lesions (76.9%, 95%CI 46.2, 95.0) compared to those without neck lesions (0%, 95%CI 0, 84.2) (Fisher's exact test p=0.095). There was also no difference in the presence of hepatomegaly in adult dolphins with neck lesions compared to adults without neck lesions (Fisher's exact test p=0.1).

5.3.2 Microbiology results

5.3.2.1 Microorganisms cultured in dolphins with and without neck lesions

Bacteria were successfully cultured from samples from 15 of the 16 cases, with all samples from Case 8 being culture-negative (Table 5-6). In total, 13 bacterial species were cultured from the 16 cases, 10 from one or more sample sites from dead dolphins with neck lesions, and seven from samples from the three dead dolphins without neck lesions (Table 5-7). One dolphin (Case 13) without neck lesions also had a fungal infection. 31% (95%CI 11.0, 58.7) of all cases (n=5) had only one bacterial species cultured from samples collected. These species were either *A. hydrophila* (n=3) or *P. shigelloides* (n=2). 63% (95%CI 35.4, 84.8) of all cases (n=10) had two or more species of bacteria cultured.

Overall the most frequent bacteria isolated from all 16 dolphins were *A. hydrophila* (50%, 95%CI 24.7, 75.3, n=8), *P. shigelloides* (38%, 95%CI 15.2, 64.6, n=6), and ß-haemolytic Group C *Streptococcus* spp. ß-haemolytic Group D *Streptococcus* spp. and *Escherichia coli* (all 19%, 95%CI 4, 45.6, n=3) (Table 5-7). The three most frequent bacteria cultured in the 13 dolphins with neck lesions were *A. hydrophila* (54%, 95%CI 25.1, 80.8, n=7), *P. shigelloides* (46%, 95%CI 19.2, 74.9, n=6), and ß-haemolytic Group C *Streptococcus* spp. (23%, 95%CI 5, 53.8, n=3). The two most frequent bacteria isolated in the dolphins with no

neck lesions were ß-haemolytic Group D *Streptococcus* spp. and *E. coli* (both 67%, 95%CI 9.4, 99.2, n=2) and these were not found to be significantly different to the dolphins with neck lesions (Fisher's exact test p=0.07).

There was no statistical difference in the isolation of *A. hydrophila* in cases with neck lesions (54%, 95%CI 25.1, 80.8) and cases without neck lesions (33.33%, 95%CI 0.8, 90.6) (OR 1.71, 95%CI 0.12, 24). Overall there was no statistically significant difference in the number of dolphins with neck lesions from which *A. hydrophila*, *P. shigelloides* or ß-haemolytic Group C *Streptococcus* spp. were cultured, compared with dolphins without neck lesions (p=0.5, 0.21, 0.51, respectively) (Table 5-7). Similarly, there were no significant differences for each of the cultured bacteria from all samples in dolphins with neck lesions compared to those without neck lesions (all p≥0.07).

Table 5-6: Culture of microbes from any of the sample sites/tissues from the individual 16 dolphins cultured in this study

	Neck lesion present													**Neck lesion absent**		
Case number	1	2	3	4	5	6	7	8	9	10	12	15	16	11	13	14
Age Class	C	C	A	C	C	C	C	J	A	J	C	C	A	A	J	A
Frozen (weeks)					2	2	4	8							12	12
Aeromonas hydrophila	+	+	-	+	-	-	+	-	+	-	+	-	+	+	-	-
Plesiomonas shigelloides	-	-	-	+	+	+	+	-	-	-	-	+	+	-	-	-
ß-haemolytic Group C *Streptococcus* spp.	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-
ß-haemolytic Group D *Streptococcus* spp.	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+
Escherichia coli	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+
Klebsiella pneumoniae	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
Enterobacter cloacae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Edwardsiella tarda	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Citrobacter freundii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Enterobacter aerogenes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Proteus vulgaris	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Proteus mirabilis	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Morganella morganii	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
Non-albicans *Candida* spp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-

A: Adults, J: Juveniles, C: calves. + Present; - Absent.

Table 5-7: Summary of the culture of microbes from the individual 16 dolphins cultured in this study

	Neck lesion present n=13		**Neck lesion absent n=3**	
	Total	% (95%CI)	Total	% (95%CI)
Aeromonas hydrophila	7	54 (25.1, 80.8)	1	33 (0.8, 90.6)
Plesiomonas shigelloides	6	46 (19.2, 74.9)	0	0 (0, 70.8)
ß-haemolytic Group C *Streptococcus* spp.	3	23 (5.0, 53.8)	0	0 (0, 70.8)
ß-haemolytic Group D *Streptococcus* spp.	1	8 (0.2, 36)	2	67 (9.4, 99.2)
Escherichia coli	1	8 (0.2, 36)	2	67 (9.4, 99.2)
Klebsiella pneumoniae	1	8 (0.2, 36)	1	33 (0.8, 90.6)
Enterobacter cloacae	0	0 (0, 24.7)	1	33 (0.8, 90.6)
Edwardsiella tarda	1	8 (0.2, 36)	0	0 (0, 70.8)
Citrobacter freundii	0	0 (0, 24.7)	1	33 (0.8, 90.6)
Enterobacter aerogenes	0	0 (0, 24.7)	1	33 (0.8, 90.6)
Proteus vulgaris	1	8 (0.2, 36)	0	0 (0, 70.8)
Proteus mirabilis	1	8 (0.2, 36)	0	0 (0, 70.8)
Morganella morganii	1	8 (0.2, 36)	0	0 (0, 70.8)
Non-albicans *Candida* spp.	0	0 (0, 24.7)	1	33 (0.8, 90.6)

5.3.2.2 Bacteria isolated from neck muscle tissue samples

The presence/absence of bacteria were compared in samples from affected neck muscle (n=13) and normal appearing neck muscle from those dolphins without any neck lesions (n=3). Bacteria were cultured from 11 of the dolphins with neck lesions (85% 95%CI 54.6, 98.1), and in all of the three without neck lesions (p=0.65). The culture results are summarised in Figure 5-4 and Table 5-8 and Table 5-9.

There was a significant difference in the culture of ß-haemolytic Group D *Streptococcus* spp. (p=0.025) and *E. coli* (p=0.025) from neck muscle samples from dolphins with neck lesions (n=13) and neck muscle samples in dolphins without evidence of neck lesions (n=3). All other bacteria cultured were not significantly different between the two groups ($p \geq 0.18$).

There was no significant difference in the bacteria cultured between samples of muscle from neck lesions and apparently normal muscle adjacent to the area with lesions in individual dolphins (p=1).

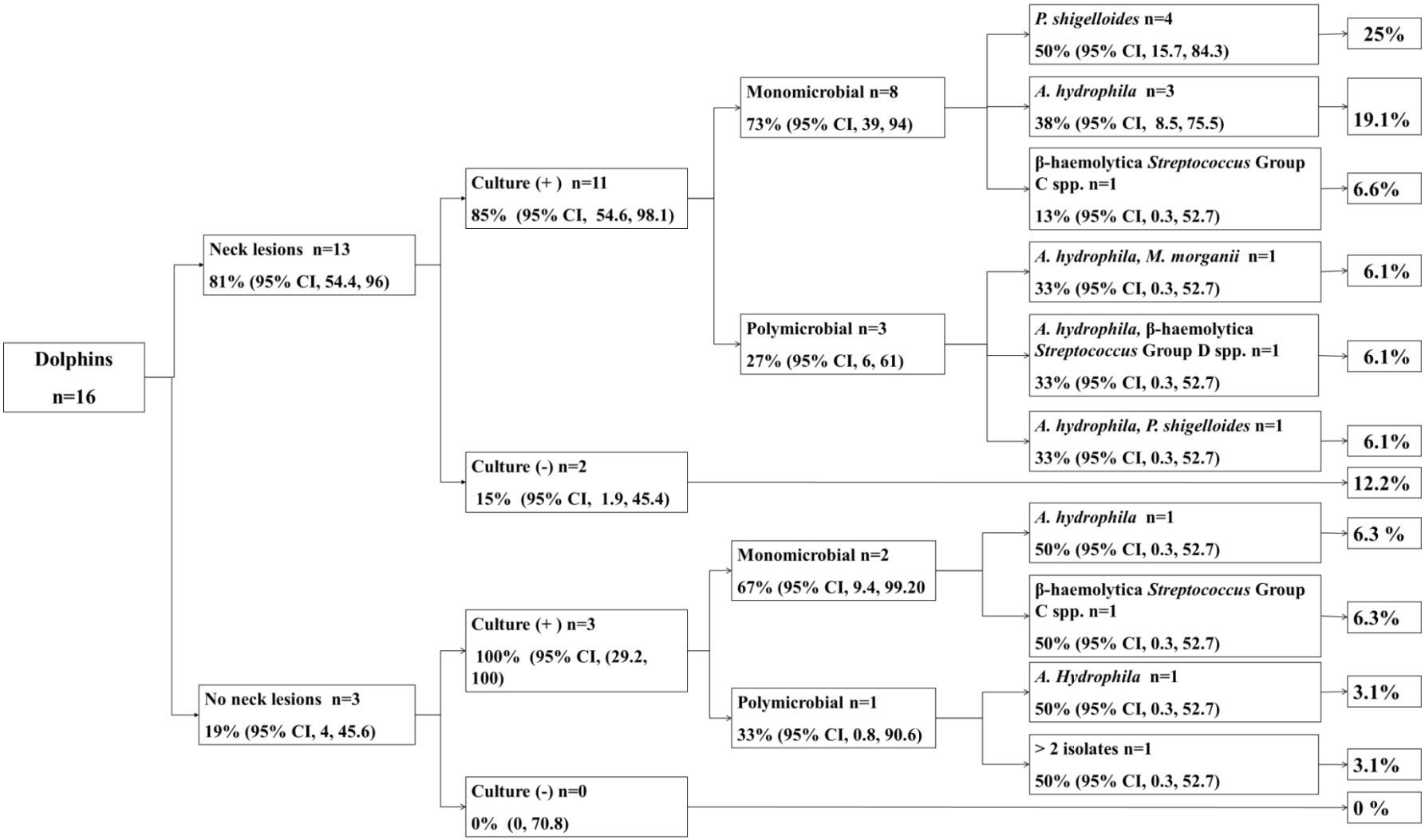


Figure 5-4: Percentage of different culture results in neck muscle in dolphins with and without neck lesions in this study.

Table 5-8: Summary of bacterial species cultured in neck muscle, grossly normal muscle adjacent to neck lesions and liver in 16 cases

		Dolphins with a neck lesion n=13													**Dolphins without a neck lesion n=3**				
	Case Number	1	2	3	4	5	6	7	8	9	10	12	15	16	11	13	14	Total cases	%
Neck muscle	*Aeromonas hydrophila*	+	+	-	+	-	-	-	-	+	-	+	-	+	+	-	-	7	43.75
	Plesiomonas shigelloides	-	-	-	+	+	+	+	-	-	-	-	+	-	-	-	-	5	31.25
	ß-haemolytic Group C *Streptococcus* spp.	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	2	12.5
	ß-haemolytic Group D *Streptococcus* spp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	2	12.5
	Escherichia coli	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	2	12.5
	Citrobacter freundii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	1	6.25
	Enterobacter aerogenes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	1	6.25
	Klebsiella pneumoniae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	1	6.25
	Morganella morganii	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	6.25
	Enterobacter cloacae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0
Normal muscle adjacent to neck lesion	*Aeromonas hydrophila*		+	-		-		-	-	+	-	+		+				4	44.4
	Plesiomonas shigelloides		-	-		+		+	-	-	-	-		-				2	22.2
	ß-haemolytic Group C *Streptococcus* spp.		-	+		-		-	-	-	-	-		+				2	22.2
	ß-haemolytic Group D *Streptococcus* spp.		-	-		-		-	-	-	-	-		-				0	0
	Escherichia coli		-	-		-		-	-	-	-	-		-				0	0

		Dolphins with a neck lesion n=13													Dolphins without a neck lesion n=3				
	Case Number	1	2	3	4	5	6	7	8	9	10	12	15	16	11	13	14	Total cases	%
	Citrobacter freundii		-	-		-		-	-	-	-	-		-				0	0
	Enterobacter aerogenes		-	-		-		-	-	-	-	-		-				0	0
	Klebsiella pneumoniae		-	-		-		-	-	-	-	-		-				0	0
	Morganella morganii		-	-		-		-	-	-	-	-		-				0	0
	Enterobacter cloacae		-	-		-		-	-	-	-	-		-				0	0
Liver	*Aeromonas hydrophila*		+			-	-	-	-	+	-	+		+	+		-	5	45.5
	Plesiomonas shigelloides		-			+	+	-	-	-	-	-		-	-		-	2	18.2
	ß-haemolytic Group C *Streptococcus* spp.		-			-	-	-	-	-	-	-		-	-		-	0	0
	ß-haemolytic Group D *Streptococcus* spp.		-			-	-	-	-	-	-	-		-	-		-	0	0
	Escherichia coli		-			-	-	-	-	-	-	-		-	-		+	1	9.1
	Citrobacter freundii		-			-	-	-	-	-	-	-		-	-		-	0	0
	Enterobacter aerogenes		-			-	-	-	-	-	-	-		-	-		-	0	0
	Klebsiella pneumoniae		-			-	-	-	-	-	-	-		-	-		-	0	0
	Morganella morganii		+			-	-	-	-	-	-	-		-	-		-	1	9.1
	Enterobacter cloacae		-			-	-	-	-	-	-	-		-	-		-	0	0

Black: no sample tested, + present, -Absent.

Table 5-9: Overall summary data of bacterial species cultured in grossly abnormal neck muscle, grossly normal neck muscle, and liver in cases with neck lesions compared to cases with no neck lesions

		Dolphins with a neck lesion			Dolphins without a neck lesion	
		Affected neck muscle	**Adjacent muscle**	**Liver**	**Neck muscle**	**Liver**
		n=13	**n=9**	**n=9**	**n=3**	**n=2**
Aeromonas hydrophila	n	6	4	4	1	1
	% (95%CI)	46.2 (19.2, 74.9)	44.4 (13.7, 78.8)	44.4 (13.7, 78.8)	33.3 (0.8, 90.6)	50 (1.3, 98.7)
Plesiomonas shigelloides	n	5	2	2	0	0
	% (95%CI)	38.5 (13.9, 68.4)	22.2 (2.8, 60)	22.2 (2.8, 60)	0 (0, 70.8)	0 (0, 84.2)
ß-haemolytic Group C *Streptococcus* spp.	n	2	2	0	0	0
	% (95%CI)	15.4 (1.9, 45.4)	22.2 (2.8, 60)	0 (0, 33.6)	0 (0, 70.8)	0 (0, 84.2)
ß-haemolytic Group D *Streptococcus* spp.	n	0	0	0	2	0
	% (95%CI)	0 (0, 24.7)	0 (0, 33.6)	0 (0, 33.6)	66.7 (9.4, 99.2)	0 (0, 84.2)
Citrobacter freundii	n	0	0	0	1	0
	% (95%CI)	0 (0, 24.7)	0 (0, 33.6)	0 (0, 33.6)	33.3 (0.8, 90.6)	0 (0, 84.2)
Enterobacter aerogenes	n	0	0	0	1	0
	% (95%CI)	0 (0, 24.7)	0 (0, 33.6)	0 (0, 33.6)	33.3 (0.8, 90.6)	0 (0, 84.2)

		Dolphins with a neck lesion			Dolphins without a neck lesion	
		Affected neck muscle	**Adjacent muscle**	**Liver**	**Neck muscle**	**Liver**
		n=13	**n=9**	**n=9**	**n=3**	**n=2**
Klebsiella pneumoniae	n	0	0	0	1	0
	% (95%CI)	0 (0, 24.7)	0 (0, 33.6)	0 (0, 33.6)	33.3 (0.8, 90.6)	0 (0, 84.2)
Escherichia coli	n	0	0	0	2	1
	% (95%CI)	0 (0, 24.7)	0 (0, 33.6)	0 (0, 33.6)	66.7 (9.4, 99.2)	50 (1.3, 98.7)
Morganella morganii	n	1	0	1	0	0
	% (95%CI)	7.7 (0.2, 36)	0 (0, 33.6)	11.1 (0.3, 48.2)	0 (0, 70.8)	0 (0, 84.2)

NL: neck lesion

Three cases whose bladder contained urine had cystocentesis performed and microbial urinalysis carried out. Case 9 had a leucocytosis (80mm^3, ref <10) with a sterile culture, Case 14 had a mild leucocytosis (14mm^3) with *E. coli*, ß-haemolytic Group D *Streptococcus* spp., *E. aerogenes*, and *C. freundii* cultured, and Case 16 had a leucocytosis (92mm^3) with *A. hydrophila* cultured.

5.3.3 Cytology results

5.3.3.1 Comparison of samples of dolphins with and without neck lesions

The leucocyte/white blood cell (WBC) count in affected muscle tissue was greater for dolphins with neck lesions (median=2) than in normal neck muscle tissue from the three dolphins without neck lesions (median=1) (Mann-Whitney U=4.5, p=0.019; Table 5-10 and Table 5-11). The WBC count was also greater in apparently normal muscle adjacent to a neck lesion in dolphins with neck lesions (median=2) than for grossly normal muscle from dolphins without neck lesions (median=1) (Mann-Whitney U=3, p=0.019). All other comparisons of bacterial and cell counts between dolphins with and without lesions were not statistically different (epithelial cells p=0.19; Gram –ve bacilli p=0.14, Gram +ve bacilli p=0.16, Gram +ve cocci p=0.14).

A Wilcoxon Signed-Rank test for paired samples (n=9) found no significant difference in the results from cytology between samples of muscle from neck lesions and apparently normal muscle adjacent to the area with lesions in individual dolphins (epithelial cells p=0.39; WBCs p=0.4, Gram –ve bacilli p=0.4).

Table 5-10: Cytology and culture results in grossly abnormal neck muscle, grossly normal muscle adjacent to the lesion and liver in 16 cases

Case #	Sample tested	Cytology		Microscopy			Bacterial colony growth	
		Epithelial cells	Leucocytes (WBCs)	Gram -ve bacilli	Gram +ve bacilli	Gram +ve cocci	Score	Species cultured
1	Neck lesion muscle	+	++	+++	-		3+	*A. hydrophila*
2	Neck lesion muscle	+	+	+++	+++		3+ 3+ 0	*A. hydrophila* *M. morganii* *Clostridium* spp.
	Normal muscle	+	++	+++	+++		3+ 3+ 0	*A. hydrophila* *M. morganii* *Clostridium* spp.
	Liver	+	+	+++	+++		3+ 3+ 0	*A. hydrophila* *M. morganii* *Clostridium* spp.
3	Neck lesion muscle	+	++	++		++	3+	ß-haemolytic Group C *Streptococcus* spp.
	Normal muscle	+	++	+	++	-	3+	ß-haemolytic Group C *Streptococcus* spp.
4	Neck lesion muscle	++	++	++	+++	-	3+ 3+	*A. hydrophila* *P. shigelloides*
5	Neck lesion muscle	+	+++	+		+	3+	*P. shigelloides*
	Normal muscle	+	+++	+		+	3+	*P. shigelloides*
	Liver	+	+++	++		++	2+	*P. shigelloides*
6	Neck lesion muscle	+	++++	+++	+	+	3+	*P. shigelloides*
	Liver	+	+++	+		+	3+	*P. shigelloides*

Case #	Sample tested	Cytology		Microscopy			Bacterial colony growth	
		Epithelial cells	Leucocytes (WBCs)	Gram -ve bacilli	Gram +ve bacilli	Gram +ve cocci	Score	Species cultured
7	Neck lesion muscle	+	+++	+	++++	+	2+	*P. shigelloides*
	Normal muscle	+	+++	+	++++	+	2+	*P. shigelloides*
	Liver	+	++	+	++++	+	0	No pathogens cultured
8	Neck lesion muscle	+++	+++	+			0	No pathogens cultured
	Normal muscle	++	++	+			0	No pathogens cultured
9	Neck lesion muscle	++	++	+	++		3+	*A. hydrophila*
	Normal muscle	++	++	+	+		2+	*A. hydrophila*
	Liver	+	++	-	+++	+	3+ 3+	*E. coli* ß-haemolytic Group C *Streptococcus* spp.
10	Neck lesion muscle	++	++	++	++	++	0	No pathogens cultured
	Normal muscle	++	++	++	++	++	0	No pathogens cultured
	Liver	++	++	++	++	++	0	No pathogens cultured
11	Neck muscle	++	++	+++	+	+++	3+	*A. hydrophila*
	Normal muscle	++	++	+		+	3+	*A. hydrophila*
	Liver	++	++	+++	+++	++	3+	*A. hydrophila*
12	Neck lesion muscle	+	++	++	++		3+	*A. hydrophila*
	Normal muscle	++	++++	+++	+++		3+	*A. hydrophila*
	Liver	+++	+++	++	+++		3+	*A. hydrophila*

Case #	Sample tested	Cytology		Microscopy			Bacterial colony growth	
		Epithelial cells	**Leucocytes (WBCs)**	**Gram -ve bacilli**	**Gram +ve bacilli**	**Gram +ve cocci**	**Score**	**Species cultured**
13	Normal muscle	0	0	+++	+++	++	3+ 3+	*E. coli* ß-haemolytic Group D *Streptococcus* spp.
	Normal muscle	+	0	+++	+	+++	3+ 3+	ß-haemolytic Group D *Streptococcus* spp. *E. coli*
14	Neck muscle	+	+	+++	++	+	3+ 3+	*C. freundii* *E. aerogenes*
	Neck muscle	0	0	+++	+	+++	3+ 3+	ß-haemolytic Group D *Streptococcus* spp. *E. coli*
	Normal muscle	+	+	++++	+++	+	3+ 3+	*K. pneumoniae* *E. coli*
	Liver	+	+	+++	-	-	3+ 3+	*K. pneumoniae* *E. coli*
15	Neck lesion muscle	++	+++	++	+	+	3+	*P. shigelloides*
16	Neck lesion muscle	++	+++	++++	++	+	3+ 3+	*A. hydrophila* ß-haemolytic Group C *Streptococcus* spp.
	Normal muscle	++	++	+++	++	+++	3+ 3+	*A. hydrophila* ß-haemolytic Group C *Streptococcus* spp.
	Liver	++	+++	+++	+++	-	3+	*A. hydrophila*

0 absent; 1+ rare; 2+ some; 3+ numerous; -not reported; Black: no sample tested.

Table 5-11: Summary data for cytology between cases with neck lesions and cases with no visible neck lesions

	Neck lesion present					**Neck lesion absent**				
	Cytology		**Microscopy**			**Cytology**		**Microscopy**		
	EC	**WBC**	**Gram -ve bacilli**	**Gram +ve bacilli**	**Gram +ve cocci**	**EC**	**WBC**	**Gram –ve bacilli**	**Gram +ve bacilli**	**Gram +ve cocci**
Neck muscle samples (n)	13	13	13	13	13	3	3	3	3	3
+ve samples (n)	13	13	13	9	7	3	3	3	3	3
% (95%CI)	100 (75.3, 100)			69 (38.6, 90.9)	54 (25.1, 80.8)	100 (29.2, 100)				
Mean	1.54	2.46	2.08	2.22	1.29	1.17	1	2.67	1.5	1.83
Median	1	2	2	2	1	1	1	3	2	2
Adjacent muscle samples (n)	9	9	9	9	9					
Samples +ve (n)	9	9	9	7	4					
% (95%CI)	100 (66.4, 100)			78 (40, 97.2)	44 (13.7, 78.8)					
Mean	1.56	2.44	1.78	2.43	1.75	1.33	1	2.67	2	1.67
Median	2	2	1	2	1.5	1	1	3	2	1

	Neck lesion present					Neck lesion absent				
	Cytology		Microscopy			Cytology		Microscopy		
	EC	**WBC**	**Gram -ve bacilli**	**Gram +ve bacilli**	**Gram +ve cocci**	**EC**	**WBC**	**Gram –ve bacilli**	**Gram +ve bacilli**	**Gram +ve cocci**
Liver samples (n)	8	8	8	8	8	2	2	2	2	2
Samples +ve (n)	8	8	7	6	5	2	2	2	1	1
% (95%CI)	100 (63.1, 100)		88 (47.3, 99.7)	75 (34.9, 96.8)	56 (21.2, 86.3)	100 (15.8, 100)			50 (1.3, 98.7)	
Mean	1.5	2.38	2	3	1.4	1.5	1.5	3	3	2
Median	1	2.5	2	3	1	1.5	1.5	3	3	2

EC: Epithelial Cells; WBC: White blood cells/ leucocytes.

5.3.3.2 Histological assessment

Of the nine cases submitted for histological assessment, partial results were available for only seven cases in CS2, with two cases (Case 10 and 12) in CS3 being too autolysed for any meaningful interpretation. Kuiken (2010 pers. comm.) only commented on marked histopathological changes that were visible to him. The main histological findings relevant to this study are summarised below with a full summary provided in Appendix 6.

Cases with neck lesions:

Case 4: Liver had multifocal fibrin deposits.

Case 5: Skin lesions had inflammatory cells (lymphocytes, plasma cells, macrophages), and the liver parenchyma had multiple large undefined round spaces.

Case 6: Laryngeal wall had abundant lymphocytes around the submucosal glands, and neck lesion muscle had muscle fibres separated by empty spaces (possibly gas), with many bacilli present.

Cases without neck lesions:

Case 11: Skin lesions had neutrophils and focal haemorrhage under the dermis, the liver had peri-portal hepatocytes with cytoplasmic fat vacuoles, and the peri-acinar hepatocytes were hypocellular containing dark pink cytoplasm. The splenic lymphoid follicles were hypocellular.

Case 14: There were neutrophils in the pia mater, and the liver had possible evidence of an inflammatory lesion, however this was undefined.

5.4 Discussion

5.4.1 Key findings to support the diagnosis of necrotising fasciitis

From the results of this study it is concluded that necrotising fasciitis is the most likely diagnosis for the neck lesions seen in the MRIDs. This conclusion was made based on a number of key findings namely: gross pathological lesions; bacterial cultures; high number of calf mortalities; hepatomegaly; and cytology. These are discussed below in more detail.

5.4.1.1 Gross pathological lesions

The gross pathological changes seen in this study are consistent with those reported in necrotising fasciitis lesions of humans (Dale et al., 1999; Henrich et al., 1995; Kaddour and Smelt, 1992; Thomas and Meyer, 2012; Vaid et al., 2002; Wolf et al., 2010; Shindo et al., 1997). In this study, the neck lesions in the MRIDs varied in size, colour and extent of lesion progression, as was demonstrated in Chapter 3 (Figure 3-2). Lesions associated with necrotising fasciitis in humans can also vary widely (Dale et al., 1999; Maynor and Kulkarni 2011; Shindo et al., 1997). Ozturk et al. (2005) described the initial skin presentation as ranging from a minor rash to erythema, oedema, induration or cellulitis. In the early stages of the infection in humans, extensive muscle and skin necrosis is not common (Dale et al., 1999), however as the disease progresses, marked tissue oedema occurs (Krebs et al., 2001), particularly associated with infections of ß-haemolytic *Streptococcus* spp. (Brook and Frazier, 1995). The oedema facilitates the rapid spread of bacteria into the fascial planes, leading to a decreased supply of oxygen and nutrients, causing vascular thrombosis (Krebs et al., 2001; Trent and Kirsner, 2002), which results in the necrosis of subcutaneous tissue, musculature and epidermis (Krebs et al., 2001, (Dale et al., 1999). At this stage blisters and ulcers may be seen, which are late findings in the disease's progression and indicate dermal

ischaemic necrosis (Shindo et al., 1997). As necrotic tissue begins to appear the compromised area becomes increasingly demarcated from normal tissue (Bain, 1999), with the skin turning purple or purple-black as tissue necrosis progresses (Trent and Kirsner, 2002). Myonecrosis also develops in the underlying musculature (Trent and Kirsner, 2002). This pathophysiological process explains the variation seen in the neck lesions of humans and, most likely, also the MRID carcasses examined in this study.

Cervical necrotising fasciitis is a life-threatening condition in humans that progresses rapidly resulting in a high case fatality rate (Allender et al., 2009; Dale et al., 1999; Shindo et al., 1997). In this study, lesions comparable with necrotising fasciitis were also found to have a predilection for the neck in MRIDs, and given the similarities with the condition in humans are likely to be a major reason for the resultant death in the affected dolphins.

5.4.1.2 Bacterial cultures

In this study, bacteria were commonly cultured from samples of neck lesions, occurring as both mono-microbial and poly-microbial infections. In humans, necrotising fasciitis can involve a range of causative bacteria, either as poly-microbial (Brook and Frazier, 1995; Dale et al., 1999; Maynor and Kulkarni, 2011; Wong et al., 2003), or mono-microbial infections. A study by Gangopadhyay et al. (2008) found that 66% of 16 necrotising fasciitis cases in human neonates were mono-microbial. However, in the current study there was no statistical difference between the presence of mono and poly-microbial infections found in dolphins with neck lesions compared to dolphins that did not have neck lesions.

Positive culture results were obtained in 88% (n=14) of the dolphins in this study, including 84% (n=11) of dolphins with neck lesions and all dolphins without neck lesions. This was similar to that reported in a study by Pereira et al. (2008), where 88% of 26 cetaceans (including five dolphin and five whale species) stranded or caught in fishing nets were culture positive. In the current study, the three principal pathogens that were cultured from the neck lesions of affected dolphins were *A. hydrophila* (54%), *P. shigelloides* (46%) and ß-haemolytic *Streptococcus* spp. (23%). *Aeromonas hydrophila* and *P. shigelloides* are both commonly reported commensals of dolphins. In the aforementioned study by Pereira et al. (2008), 176 swabs were collected and cultured resulting in the detection of 114 bacterial strains/species from the 26 cetaceans sampled. Of the bacteria cultured, *A. hydrophila* and *P. shigelloides* were cultured from seven cetacean species. Similarly, Buck et al. (2006) cultured *A. hydrophila* and *P. shigelloides* from free-ranging (presumably healthy) bottlenose dolphins (*T. truncatus*).

In this study, there were no significant differences found in the bacteria cultured from affected neck lesion tissue and adjacent, apparently normal looking, muscle tissue from the same animals. This is likely due to two reasons: firstly, these bacteria are commensals; and secondly, the causative bacteria in necrotising fasciitis are correlated with their presence as normal flora in the body tissues adjacent to the site of the lesion (Brook and Frazier, 1995).

5.4.1.2.1 *Aeromonas hydrophila*

Aeromonas hydrophila has frequently been cultured from necrotising fasciitis in humans (Apisarnthanarak et al., 2008; Arroyo et al., 2010; Markov et al., 2007; Minnaganti et al., 2000; Monaghan et al., 2008) and has been associated with diet. For example, in a study by

Goh et al. (2014) they found that liver disease was a risk factor for the condition in some parts of Asia in people who consumed fish/seafood, in addition, infection arising subsequent to trauma, due to fish fins was commonly associated with both *Vibrio* spp., and *Aeromonas* spp. infections.

5.4.1.2.2 *Plesiomonas shigelloides*

Members of the genus *Plesiomonas* have not been linked with necrotising fasciitis in humans, despite *P. shigelloides* being cultured from traumatic wounds (Janda et al., 2016). Although not significant in the current study, *P. shigelloides* was more frequently cultured from affected neck muscle than from apparently normal adjacent muscle in individual dolphins, and from dolphins with neck lesions than those without neck lesions.

5.4.1.2.3 *Streptococcus* spp.

In this study, ß-haemolytic *Streptococcus* Group C spp. were isolated from two cases with neck lesions (Cases 3, 16) but from no cases without neck lesions. Necrotising fasciitis is commonly associated with *Streptococcus* spp. in humans (Bishop et al., 2007; Gaunt et al., 1984; Hever et al., 2016; Kittang et al., 2017; Kulendra and Corr, 2008; Meleney, 1924; Nolff and Meyer-Lindenberg, 2015; Shindo et al., 1997; Wong et al., 2003; Zappulli et al., 2005), however most infections in humans with ß-haemolytic Group C *Streptococcus* spp. occur in people with significant underlying conditions (Hever et al., 2016; Korman et al., 2004), and reports of the involvement of this Lancefield group with necrotising fasciitis is less commonly reported (Gaunt et al., 1984; Hever et al., 2016; Kittang et al., 2017; Korman et al., 2004) than with infections with Group A streptococci.

5.4.1.3 Calf mortality

In this study, there were more dead MRID calves with neck lesions than adults. This may be linked to the increased risk of necrotising fasciitis in younger animals and its higher case fatality, as has also been reported in an outbreak of necrotising fasciitis in young crocodiles (*Crocodylus porosus*) (Bishop et al., 2007). However, in humans necrotising fasciitis has resulted in high mortality (Cui et al., 2007; Diab et al., 2020; Shindo et al., 1997; Thomas and Meyer, 2012) in all age groups (Gangopadhyay et al., 2008; Hsieh et al., 1999; Krebs et al., 2001; Ozturk et al., 2005; Sandell and Ramanan, 2000), with no specific reported predilection for neonates.

5.4.1.4 Hepatomegaly

Dolphins with neck lesions in this study were eight times more likely to have hepatomegaly, although the analysis was not significant as it lacked power with only two cases in the no neck lesion group for comparison. Liver disease has been shown to be a predisposing factor for necrotising fasciitis in humans (Dale et al., 1999; Hung et al., 2014; Shindo et al., 1997; Wong et al., 2003).

The histopathological results of four cases in this study suggest some underlying liver pathology, with hepatic lipidosis confirmed in one case and fibrin deposits in another (Lisman and Jenne, 2018). Fatty liver disease in bottlenose dolphins (*T. truncatus*) has been linked to non-hereditary metabolic disorders (Venn-Watson et al., 2012). Furthermore, the aetiology of hepatomegaly can also include infections, primary liver disease, diabetes mellitus, and toxicities from contaminants such as chlorinated compounds, heavy metals and phenols (Tilley and smith, 2011). The potential role of contaminants will be examined further

in this thesis in Chapter 7. Although the aetiology of the liver pathology in the cases in this study remain largely unknown, the hepatomegaly found in the MRID population, together with the histological results, may indicate an underlying pathogenesis that may, in part, contribute to the emergence of necrotising fasciitis in this population. Further studies are required to validate this.

5.4.1.5 Cytology

The results of cytology in this study indicate an inflammatory process in dolphins with neck lesions. There was a significant difference between the number of leucocytes present in the dolphin cases with neck lesions and those without neck lesions. Similarly, there was a significant difference when comparing neck muscle in dolphins without neck lesions to the presumably healthy muscle adjacent to the neck lesions in affected dolphins. This would be expected if an infectious agent was associated with the aetiology of the neck lesions.

5.4.2 Incidental findings

In addition to the key findings, there were three incidental findings in this study.

5.4.2.1 Skin lesions

In this study, 15 of the 16 dolphins had skin lesions (other than those of the neck lesions) ranging from dermal pitting, minor ulcerative lesions and tattoo like lesions to severe teeth rake marks most likely from conspecifics. Case 3 was the only dolphin without skin lesions. Dale et al. (1999) reviewed cases of humans with necrotising fasciitis in the head and neck region, and reported that the majority of infections were usually the result of some minor

traumatic event, such as from an insect bite, minor cut, blunt trauma, or abrasion. Dove et al. (2007) revealed photographic evidence of social behaviour in the MRIDs, where they were seen to bite each other on the head and neck. These behaviours may have led to the traumatic skin lesions observed, and the lesions then facilitated the development of necrotising fasciitis in susceptible dolphins by providing a potential entry portal for opportunistic bacteria. Further studies are required to assess the validity of this hypothesis.

5.4.2.2 Stomach contents

In this study, only one (Case 9) of 13 cases with neck lesions and one (Case 14) of two cases without neck lesions had stomach contents at necropsy. This finding may indicate that the majority of the dolphins were either sick or were physically unable to forage (mature dolphins) or suckle (calves) (Baker et al., 2006). Although dolphins that have died from entanglement in fishing gear or from boat strikes normally contain food contents in their stomachs (Marçalo et al., 2021; Milani et al., 2018), Jepson et al. (2013) demonstrated that in unusual mass stranding mortality events the stomachs of dolphins are often empty.

5.4.2.3 Pharyngeal infection

In the histological analysis conducted as part of this study, an inflammatory process was detected in the submucosal glands of the laryngeal wall of Case 6. Dental or pharyngeal infection that extend to involve the deep cervical soft tissue spreading along the deep fascial planes, has been documented in several human cases of cervical necrotising fasciitis (Dale et al., 1999; Shindo et al., 1997). The current finding may suggest that, in this particular case, inflammation in the larynx may have served as an entry portal for *P. shigelloides*.

5.4.2.4 Treatment of necrotising fasciitis

In this study, a high proportion of dead MRIDs, particularly calves, had lesions similar to those observed in necrotising fasciitis of humans. In a study by Tang et al. (2006), prophylactic antimicrobials were administered to human patients with a known risk of developing necrotising fasciitis from a specific injury induced by a stonefish (*Synanceia horrida*) sting, to minimise the likelihood of developing the disease. Successful treatment in humans involves early diagnosis, antimicrobial administration, aggressive surgical debridement and supportive therapy (Shindo et al., 1997), as left untreated the case fatality rate in humans can be as high as 70% (Trent and Kirsner, 2002). Dolphins are able to mask illness, making early diagnosis and treatment difficult. For example, in a case report by Zappulli et al. (2005) a bottlenose dolphin (*T. truncatus*) with necrotising fasciitis was treated for septicaemia and endotoxic shock, but died within two hours after the onset of clinical signs. Whilst surgical intervention and debridement (Escobar et al., 2005; Leiblein et al., 2018) is the treatment of choice for necrotising fasciitis in humans, such interventions, along with most therapeutic measures, are impossible to apply to wild dolphins. As such prevention is more appropriate, which requires a sound understanding of the disease and associated pre-disposing conditions. This could potentially be aided by prophylactic treatment with antimicrobials administered via darting to minimise bacterial infection in individual dolphins and implementing practices to improve the immune system of at-risk populations of dolphins. Although in human neonates it is believed that a cost-effective measure of intervention is by increasing immunity through blood transfusions (Gangopadhyay et al., 2008), this is also impractical in wild dolphins, although it has been successfully undertaken in bottlenose dolphins (*T. truncatus*) which received blood transfusions from the same dolphin species or from another species of cetacean (*Orcinus orca*) after cross-matching the blood (Walsh and Gearhart, 2001).

5.4.3 Study limitations

There were a number of limitations in this study.

Firstly, diagnosing a medical condition based on examination of historical necropsy findings alone is difficult and poses certain challenges as discussed previously in Chapter 4. In particular, post-mortem bacterial over-growth could alter the findings in this study, and so, where possible, necropsies and in particular microbiological investigations should focus on animals in CS2. Decomposition can alter both the microbiological fauna, as well as limit the ability to interpret and detect histopathological changes.

Secondly, in this study, only three dolphins were found without neck lesions for inclusion in a "control" group, one of which was not able to be necropsied completely due to refusal by the Cambodian Government. These low numbers seriously limit the power of this study, and consequently further carcasses should be collected and full necropsies conducted to generate a bigger data set.

Thirdly, histological analysis has proven to be of very limited value in the MRIDs, particularly in neck lesion samples that were severely autolysed, as discussed in Chapter 4.

Fourthly, the majority of data in the literature on necrotising fasciitis relates to humans, with only a handful of cases reported in other animals, with even fewer in dolphins, thus extrapolating findings from human medicine to those found in this study was unavoidable, although the validity of this is uncertain.

A final limitation of this study is the failure to have calves without neck lesions - this impacts the validity of the calf findings, however during the four years I performed post mortem examinations, all calves necropsied presented with neck lesions.

5.5 Conclusions

In conclusion, this is the first study diagnosing lesions analogous to necrotising fasciitis in dead MRIDs with neck lesions. This is the first description of necrotising-like lesions in wild dolphins, and the first reported cases of a condition clinically similar to necrotising fasciitis, in MRIDs.

Skin lesions were a common finding in this study of dead dolphins with neck lesions and those without neck lesions. Consequently, a study was undertaken to determine the prevalence of skin lesions in live MRIDs at the population level, and the results of this study are presented in the following chapter.

CHAPTER SIX

An epidemiological investigation of skin lesions in live Mekong River Irrawaddy dolphins using photographic methods

6.1 Introduction

In the previous chapters (4 and 5), I reported on the finding of skin lesions in a number of dead MRIDs at necropsy; however the actual prevalence of skin lesions in this population has never been reported previously. In addition, the significance of skin lesions and their implication on the health of the population has also never been described. As discussed in Chapter 4, skin lesions in dolphins have been reported in many populations globally (Gonzalvo et al., 2015; Hart et al., 2012; Harzen and Brunnick, 1997; Herr et al., 2020; Rowe et al., 2010; Taylor et al., 2021a; Van Bressem et al., 2003; Van Bressem et al., 2009; Van Bressem et al., 2014a; Van Bressem et al., 2014b) with several aetiological agents identified including fungi, bacteria, protozoa and viruses (Hart et al., 2012; Rowe et al., 2010; Van Bressem et al., 2003; Van Bressem and Van Waerebeek, 1996; Van Bressem et al., 2009; Van Bressem et al., 2014b), however the prevalence of skin lesions in freshwater dolphins, and specifically in *O. brevirostris*, has not been widely studied. Only one study on cutaneous nodules in six populations of Irrawaddy dolphins from Malaysia, India (Chilika Lake) and Bangladesh has been published (Van Bressem et al., 2014b).

Skin lesions in MRIDs may be indicative of poor health, the presence of other underlying conditions/comorbidities or the results of exposure to environmental insults present in the Mekong River. An underlying health concern may include immunosuppression (Hart et al.,

2012; Lahvis et al., 1995), as Reif et al. (2009) identified that immunosuppression predisposed *T. truncatus* to skin lesions. In the studies presented in Chapters 3 and 4, neck lesions were identified more commonly in dead dolphin calves than in dead adults, which may suggest this younger population may be more susceptible to some level of immunosuppression. Why calves are affected, in particular, in the MRID population is concerning, and at this stage remains unknown. However, in an extensive review by Rychlik and Sillé (2019), studies in humans and other animals were reported where gestational immuno-toxic exposure from environmental contaminants resulted in immunosuppression in neonates, and include: lead in rats (F344 inbred strain of *Rattus norvegicus*) (Bunn et al., 2001); cadmium in rats and chickens (*Gallus gallus domesticus*) (Holásková et al., 2012); mercury in rats (Tonk et al., 2010); arsenic in humans (Ahmed et al., 2012; Kile et al., 2014; Nygaard et al., 2017; Raqib et al., 2009); zinc in minks (*Neovison vison*) (Bleavins et al., 1983); and dioxins in mice (*Mus musculus*) (Boule et al., 2014).

Skin lesions can also be a sensitive indicator of environmental and anthropogenic impacts, with studies on wild dolphins having identified potential links between these (Deem et al., 2001; Rowe et al., 2010; Wang et al., 2018). For example, in a study by Hart et al. (2012), different geographical locations were shown to have different skin lesion types, prevalence, and anatomical distribution of the lesions in bottlenose dolphins (*T. truncatus*). These authors proposed that geographical differences in the frequency of skin lesions may be due to seasonal or environmental influences, exposure to anthropogenic influences, exposure to contaminants or differences in population demographics and susceptibility to pathogens. Hart et al. (2012) also found that there may be a lag time between exposure to these influences and clinical manifestation of disease, highlighting the need for prompt action and investigation into dolphin populations with evidence of skin lesions.

As skin lesions may be an indicator of ill-health and immunosuppression at a population level, and to further understand the occurrence of skin lesions in the MRID population, I conducted a comparative examination of the prevalence of skin lesions in MRIDs in the nine dolphin pools of a 200km section of the Mekong River. Previous studies evaluating skin lesions in dolphins have primarily used photo-identification methods, as well as through examination of stranding and bycatch data (Hart et al., 2012; Hupman et al., 2017; Rowe et al., 2010). A major limitation of examination of the retrospective necropsy data reported in the previous two chapters is that they do not provide information on the prevalence of skin lesions in the source population, nor ascertain differences between geographical locations. Thus, to address these limitations, I conducted a study of skin lesions using photo-identification (photo-id) data of live MRID collected by myself from 2007 to 2010.

6.2 Materials and methods

6.2.1 Study area

The Mekong River is a large seasonal floodplain river which includes nine deep pool areas (Beasley, 2007) which are inhabited by MRID (Beasley, 2007; Dove et al., 2008). A map of the Mekong River, together with dolphin sightings used in this study was constructed using ArcGIS software (Figure 6-1). The survey route was described previously in Ryan et al. (2011). Each survey took between nine and 11 days to complete (Table 6-1). This *O. brevirostris* population is assumed to be a geographically and demographically closed population, as all the "marked" dolphins in the Mekong River had previously been identified (Ryan et al., 2011) during the survey efforts used in the current study.

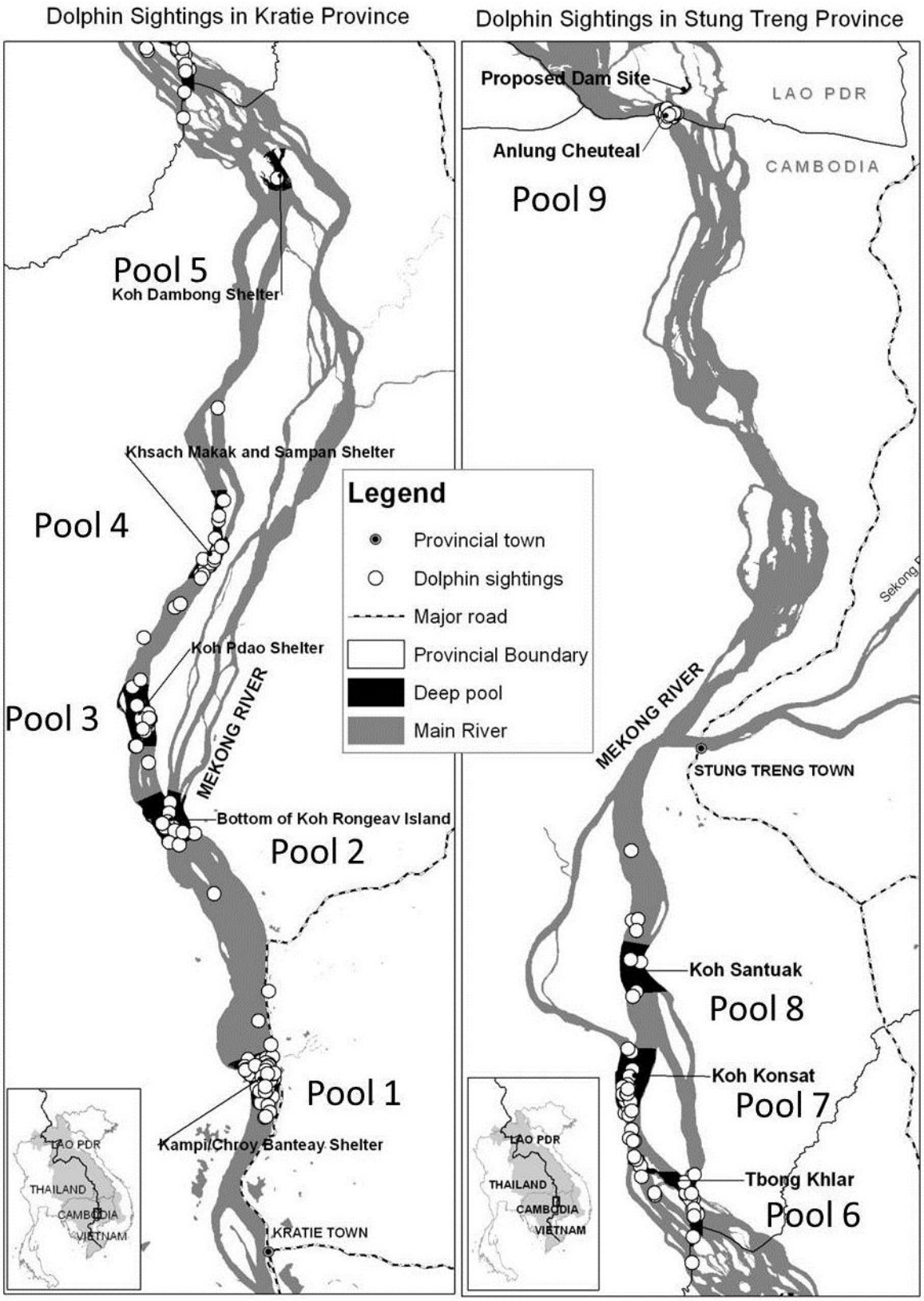


Figure 6-1: Map of the nine dolphin pools in the Mekong River, with dolphin sightings from the 11 photo-identification surveys included in this study (Ryan et al., 2011).

6.2.2 Survey methods

Prior to each survey I conducted a training workshop with my research team, to ensure all methods were consistent between surveys and that each observer understood their role on the boat. Several photography training days were conducted prior to the first survey in order to determine the best person to take photographs, and to enhance the ability of the team to take better photographs. A roster was given to the research team, assigning locations within the boat for observers and photographers for each day of the survey. Two or three photographers were assigned to each survey. Each day, one principal photographer was assigned and provided with the better quality camera and lens, and one of the ancillary photographers given the lower quality camera and lenses.

Survey methods for photo-identification were as described by Ryan et al. (2011) and Dove et al. (2008). During the surveys, the route followed a system of concrete channel-markers installed by the former French colonial government, although these markers were only visible in the low water (dry season) from October to April. Eleven surveys were conducted from April 2007 to April 2010 (Table 6-1), generally during low water when the river covers a much smaller area and dolphins were more easily observed/detected. The boat travelled at approximately 5-10 knots. Where the river was wider than 500m, a zigzag course was travelled from bank to bank to effectively cover the whole area and maximise the opportunity to sight dolphins. In addition to the driver, at least six active observers were on-board (two looking forward, two looking behind, and two looking to either side). A rotating roster and scheduled breaks were implemented to minimise fatigue of observers.

When dolphins were sighted the engine was immediately turned off to mitigate disturbance, and the driver used an oar to cautiously approach to within 100m of the dolphin(s). To further

minimise potential disturbance, the animals were approached in a parallel manner, usually stopping upstream from the group and floating past them. Where the current was particularly strong, repeatedly motoring upstream of the group and drifting past was necessary.

During each sighting dolphins were counted by all observers on the boat and this count was averaged. The dolphins were photographed by two photographers using digital cameras with large zoom lenses from a platform raised 25cm above the water in the bow of the boat. The actual number of dolphins counted was typically lower than the number confirmed from photo-identification (Table 6-1). This was due to several reasons: the dolphins do not always surface simultaneously; when groups were large it can be difficult to count each dolphin as they surface; the dolphins frequently surfaced at different locations around the boat; the dolphins often only surfaced momentarily for a breath and then returned under the water; visibility in the Mekong River is poor so counting relies on seeing dolphins come up for a breath; and finally, these dolphins are naturally very elusive. Dolphins within a sighting were photographed for a minimum of 30 minutes and a maximum of two hours to allow adequate time for good photographs to be taken whilst minimising disturbance. Generally tens of fast-action (sports-mode) photographs were taken of each dolphin as it surfaced and these photos were used to subsequently identify individuals present at each session. Observations commenced at 7am and finished prior to dusk. On each successive survey day the boat began at the place it had ended at on the preceding afternoon, incrementally working up-stream, and then down-stream within the study area.

Table 6-1: Photo-identification survey summary data (adapted from Ryan et al., 2011)

Survey						Mekong River Irrawaddy dolphins				
Year	**No.**	**Date start**	**Date finish**	**Length (days)**	**Interval[†] (days)**	**# Counted***	**# ID[~]**	**Cumulative total ID**	**Proportion ID[a] (counted)**	**Proportion ID[b] (total n=88)**
2007	1[1,2]	17 April	25 April	9	-	58	62	62	0.94	0.70
	2[2]	21 May	29 May	9	34	58	61	76	0.95	0.69
	3[2]	29 Oct	8 Nov	11	162	38	41	76	0.93	0.47
2008	4[2]	18 Feb	27 Feb	10	112	57	60	78	0.95	0.68
	5[2]	21 Apr	1 May	11	64	46	48	79	0.96	0.55
	6[2]	25 May	3 Jun	10	34	37	40	80	0.93	0.45
	7[3]	30 Nov	9 Dec	10	189	36	38	83	0.95	0.43
2009	8[3,4]	13 Mar	22 Mar	10	103	64	69	85	0.93	0.78
	9[3,4]	21 April	30 Apr	10	39	58	64	87	0.91	0.73
2010	10[5,6]	2 Mar	10 Mar	9	315	59	63	88	0.94	0.72
	11[5,6]	31 Mar	9 Apr	10	30	56	62	88	0.90	0.70

[1]Nikon D200/Nikkor 70-400 mm; [2]Canon EOS 350D/Sigma 170-500 mm; [3]Canon EOS 350D/Canon 100-400 mm; [4]Canon EOS 450D/Sigma 170-500mm; [5]Canon EOS 450D/ Canon 100-400 mm; [6]Canon EOS 50D/Canon 100-400 mm. [†]Interval: number of days between midpoints of surveys, * number of dolphins sighted, [~]# ID: the number of unique individual dolphins identified at each survey, Cumulative Total ID: the cumulative number of unique individual dolphins identified, Proportion ID[a]: the proportion of individuals counted of the number identified at each survey. Proportion ID[b]: the proportion of individuals counted at each survey of the total number identified in the 11 surveys (88).

6.2.3 Photograph identification and data collection

In this study, I used the photo-id data to estimate the type and prevalence of skin lesions in the sighted MRIDs. Approximately 220,000 images of dolphins (20,000 per survey) taken for photo-identification studies in 2007 to 2010 were examined for skin disorders. Only photographs showing the whole dorsal fin in clear focus and perpendicular to the camera lens, as demonstrated in Table 6-2, were used in the analysis. Individuals were identified based on the profile shape of the fin, supplemented by deformities, pigmentation, scarring and lesions, and compared with a catalogue database (Dove et al., 2008). High quality photographs of unmarked (un-identified) dolphins were also kept and recorded in a similar way to marked (identity confirmed) dolphins, as subtle distinctions occur among all dorsal fins, such that unmarked dolphins were able to be differentiated within the same sighting and survey. Dolphins that were identified on different occasions within a survey were only included once, so as to not overestimate the number of individuals in the population. In this study, MRIDs with skin lesions that were identified on multiple surveys were only included once each in the analysis. Data for pools 2 and 3, which are in very close proximity, were combined for the geographical analysis as dolphins moved freely between these two pools.

The prevalence of skin lesions were compared in pools subject to eco-tourism boat traffic (pools 1 and 9) to pools where no eco-tourism occurred (pools 2 - 8). In addition, the prevalence of skin lesions were compared in pools with minimal gillnet use (pools 1 - 8) to pool 9, a trans-boundary pool where gillnet use was extensive and unregulated. The prevalence of skin lesions between surveys within each individual pool (with data for pools 2 and 3 combined) were also compared.

6.2.4 Statistical analyses

Odds ratios and their 95%CI, using Woolf's method, as described by Kahn and Sempos (1989), were estimated for putative risk factors. 95%CI for proportions were calculated using the exact binomial method and 95%CI for means were calculated using a T-Table (Excel 2007). Statistical analysis for skin lesions in dolphin groups in different pools, months, and surveys were compared using Pearson's Chi-squared tests for independence or Fisher's exact tests, using the Real Statistics Resource Pack (Release 5.4) for Excel (2010) (Zaiontz, 2018).

6.3 Results

6.3.1 Types of skin lesions identified in this study

The types of skin lesions in this study varied, with large round focal lesions, generalised lesions, pallor lesions and tattoo-like lesions (similar to those reported in Chapter 4) being detected. Examples of various skin lesions observed in the MRIDs in this study are shown in Table 6-2.

Table 6-2: Examples of different skin lesions observed in the Mekong River Irrawaddy dolphin population from photo-identification

Photograph of skin lesions	**Description**
	A number of pallor lesions cranial to the dorsal fin
	Large pallor, well circumscribed, focal lesion on the caudal aspect of the dorsal fin
	Cutaneous well circumscribed nodules diffusely distributed in a generalised pattern on the dorsal fin, giving the impression of a nodular dorsal fin
	Several white small focal lesions diffusely distributed in a generalised pattern over the trunk

6.3.2 Prevalence of skin lesions by survey

In this study, the mean prevalence of dolphins photographed with skin lesions across all surveys was 7% (median 5.3%, range 1.5-17.5%; 95%CI 1.9, 16.7). The prevalence of lesions was highest in the following surveys: 10 (March 2009), 11 (April 2010), 5 (April/May 2008), and 2 (May 2007) (Table 6-3). The mean group size per sighting across all surveys was 5.6 (SD 3.95). The data for skin lesions in each pool by survey are summarised in Table 6-4.

In this study, dorsal fin identification photographs, representing 99% of the marked (adult) dolphins (n=84) (Ryan et al., 2011) and 95% of all photo-identified MRIDs (n=88), were analysed for lesion prevalence and extent. Skin lesions were found to be common in dolphins across all nine dolphin pools, being detected in 35 (41.6%; 95%CI 31.1, 52.2) individuals in the marked dolphins (n=84) on at least one point in time during the study period. Of these 35 dolphins identified with skin lesions, eight (23%; 95%CI 10.4, 40.1) had skin lesions detected on two or more different surveys, representing 9.5% (95%CI 4.2, 17.9) of the 84 marked dolphins observed.

Table 6-3: Skin lesion data for each photo-identification survey for the Mekong River Irrawaddy dolphin population

Survey number	Season	Total # ID dolphins	Skin lesions		Prevalence of skin lesions % (95%CI)	Odds Ratio (95%CI)
			Present	Absent		
1	Dry	62	3	59	5 (1.01, 13.5)	0.40 (0.1, 1.62)
2	Dry	61	5	56	8 (2.72, 18.1)	0.70 (0.21, 2.34)
3	Wet	41	2	39	5 (0.6, 16.53)	0.40 (0.08, 2.04)
4	Dry	60	1	59	2 (0.04, 16.53)	0.13 (0.02, 1.12)
5	Dry	48	4	44	8 (2.32, 19.98)	0.71 (0.2, 2.6)
6	Dry	40	2	38	5 (0.61, 16.92)	0.41 (0.08, 2.1)
7	Wet	38	2	36	5 (0.64, 17.75)	0.44 (0.09, 2.22)
8	Dry	69	1	68	1 (0.04, 7.81)	0.12 (0.01, 0.97)
9	Dry	64	4	60	6 (1.73, 15.24)	0.52 (0.15, 1.89)
10	Dry	63	11	52	17 (9.05, 29.1)	1.66 (0.6, 4.61)
11	Dry	62	7	55	11 (4.66, 21.89)	1.00

6.3.3 Prevalence of skin lesions by time of year

There was a significant difference (χ^2 (d.f. 1, 10) =19.9, p=0.03) between the number of dolphins observed with lesions in the 11 surveys. However, when comparing the presence of skin lesions by survey month across different years, there was a significantly higher prevalence in March 2009 (survey 10 – 17%) than March 2008 (survey 8 – 1%) (Fisher's exact test p=0.001), in contrast, there were no significant differences in the surveys conducted in April (surveys 1; 5; 9; 11 (χ^2 (d.f. 1, 3) =2.07, p=0.55) and in May (surveys 2 and 6 Fisher's exact test p=0.7).

6.3.4 Prevalence of skin lesions by season

There was no significant difference between the prevalence of skin lesions in the dry season (6.5%; 95%CI 1.8, 15.7) compared to the wet season (5.3%; 95%CI 0.6, 17.7) (χ^2 (d.f. 1, 1) =0.06, p=0.8; OR 1.24 95%CI 0.22, 7.13) (Table 6-3). Additionally, there was no significant difference found between the prevalence of skin lesions in the two wet season surveys (surveys 3 and 7, Fisher's exact test p=1), and between the nine dry season surveys (χ^2 (d.f. 1, 8) =6.4, p=0.09). Furthermore, there was no significant difference between the prevalence of skin lesions between years (χ^2 (d.f. 1, 3) =0.4, p=0.4).

6.3.5 Prevalence of skin lesions by geographical location

The prevalence of skin lesions in the different pools are summarised in Table 6-4 and Table 6-5. The prevalence was highest among dolphins from pools 5 and 9; with all dolphins observed in these pools having skin lesions. In contrast, none of the dolphins observed in pool 4 (0%; 95%CI 0, 36.9; n=8) had lesions. During this study, although dolphins were seen

in pool 8, they could not be satisfactorily photographed and therefore could not be identified. Consequently this pool was omitted from the analysis.

Overall there was a significant difference in the prevalence of skin lesions in dolphins between the seven pools (data for pools 2 and 3 combined, and pool 8 excluded as no dolphins photographed/identified) (χ^2 (d.f. 1, 6, n=89) =21.3, p=0.0015). There was a significant difference in the prevalence of skin lesions between pools 1 and 9 (Fisher's exact test p=0.002; OR could not be calculated as all dolphins in pool 9 were affected). However, there was no significant difference between the prevalence of skin lesions in dolphins from pool 1 and all other pools (Table 6-5).

Table 6-4: The number of dolphins observed with skin lesions in the different pools of the Mekong River during the 11 surveys

Survey number	**Pools**									
	1	**2 & 3**	**4**	**5**	**6**	**7**	**8**	**9**	**Total**	**# ID**
1	1	2	0	0	0	0	-	0	3	**62**
2	2	1	0	1	0	0	-	1	5	**61**
3	1	1	0	0	0	0	-	0	2	**41**
4	1	0	0	0	0	0	-	0	1	**60**
5	1	0	0	0	2	0	-	1	4	**48**
6	0	2	0	0	0	0	-	0	2	**40**
7	1	0	0	0	0	0	-	1	2	**38**
8	0	0	0	0	0	1	-	0	1	**69**
9	1	1	0	0	0	1	-	1	4	**64**
10	3	3	0	0	1	0	-	4	11	**63**
11	5	1	0	0	0	0	-	1	7	**62**
Total*	16	11	0	1	3	2	-	9	42	

- Unknown, *Includes dolphins that were photo-identified on different surveys, #ID: number of individual dolphins identified by photo-identification

Table 6-5: Summary of the presence of skin lesions on dolphins from the different pools of the Mekong River

Pools	Skin lesions present	Total # ID dolphins	Prevalence % (95%CI)	Odds Ratio (95%CI)
1[#*]	11	31	35.5 (19.2, 54.6)	1.0
2, 3	11	22	50 (28.2, 71.8)	1.75 (0.6, 5.10)
4	0	8	0 (0, 36.9)	0.19 (0.02, 1.73)
5	1	1	100 (2.5, 100)	-
6	3	8	37.5 (8.5, 75.5)	1.17 (0.27, 4.98)
7	2	12	16.7 (2.1, 48.4)	0.48 (0.11, 2.06)
8	0	0	-	-
9*	7	7[†]	100 (59, 100)	-
Total	35	89	39.3 (29.1, 50.3)	-

[#]Comparative group, *significantly different between pools p=0.002, [†]includes one additional dolphin that was unmarked and could be identified at each survey, but was not included in the database due to a lack of identifiable markings (n=88).

6.4 Discussion

6.4.1 Key findings

In this study, skin lesions were detected in 41.6% (95%CI 31.1, 52.2) of the 84 marked MRIDs on one or more surveys. This was significantly higher than that reported by Hart et al. (2012) in bottlenose dolphins (*T. truncatus*) from Charleston, Florida (overall 37.3%, 95%CI 29.6, 45.4, comprising resident dolphins 18% (n=86, 95%CI 10.1, 27.1) and non-resident dolphins 25% (n=77, 95%CI 15.6, 35.8)), but similar to that reported by Gonzalvo et al. (2015) also in *T. truncatus* in the Gulf of Ambracia, western Greece of 37% (n=153, 95%CI 29.6, 45.4).

The prevalence of skin lesions was slightly higher (7%) in the dry season (Oct-April), when water levels were low and temperatures very high, than in the wet season (5%) (May-Sept),

however this difference was not significant. Dolphins were also easier to locate in the dry season compared to the wet season, as the size of the Mekong River was far greater in the wet season and so the time taken to effectively cover each pool was also far greater.

In this study, skin lesions were prevalent in dolphins across all sections of the Mekong River surveyed with the exception of those from pool 4. Dolphins in pool 9 were found to have a significantly higher likelihood of skin lesions compared with dolphins observed in pool 1. Similarly, Rowe et al. (2010) also found that the prevalence of lesions in *T. truncatus* varied between geographical locations in Doubtful Sound and Dusty Sound, New Zealand, although they were part of the same geographical region. They hypothesised that skin lesions occurred as a result of factors specific to a population's habitat. The difference found in this study between pool 9 and the rest of the pools may be due to environmental factors such: as geographical isolation; exposure to anthropogenic influences, particularly gillnets; or differences in human population density surrounding the pool, as was reported in a study by Hart et al. (2012). The major apparent difference between pool 9 and the other pools is that the former pool is a geographically isolated, small pool (Beasley et al., 2014) (Figure 6-1), where dolphin movement was limited. In addition, pool 9 is the only pool where the use of gillnets was not restricted (Limsong et al., 2017; Vibol et al., 2009) and dolphin eco-tourism was unregulated. The higher detected prevalence of skin lesions of dolphins from pool 9 will be discussed in relation to these three factors:

1. Gillnets

Pool 9 is a trans-boundary pool in Cambodia and Laos PDR. The use of gillnets is illegal in the dolphin habitat range in Cambodia; in contrast their use is legal and unrestricted and hence extensive in the Laos PDR side of this pool. When comparing the overall risk of skin lesions in dolphins from pool 1 to those from other pools, the prevalence in dolphins from

pool 1 was only significantly different to dolphins from pool 9 where they were exposed to gillnets. During this study, the research team frequently witnessed dolphins removing snared fish from gillnets in this pool. Gillnets may directly lead to skin wounds, such as skin abrasions and ulcerations (Luksenburg, 2014; Wang et al., 2018), which may then indirectly facilitate transmission of environmental and opportunistic organisms (Diaz, 2014), particularly if the host's defence mechanisms are compromised (Shaw and Kennedy, 2006).

2. Eco-tourism

Beasley et al. (2014) hypothesised that eco-tourism was having an adverse effect on the dolphins in the Mekong River. Eco-tourism occurred in both pools 1 and 9, the only two pools easily accessible to tourists. In pool 1, eco-tourism is regulated by the Cambodian government; however in the trans-boundary pool 9, eco-tourism of the dolphins is controlled by the Laos PDR government, with no official regulations in place. The dolphins in pool 1 were subjected to greater intensity from eco-tourism associated motorised boats, with more than 20 boats operating simultaneously to take tourists to see the dolphins (Beasley et al., 2014). In contrast in pool 9, tourists were taken on a short boat trip to an island in the centre of the pool, from where they watched the dolphins. However, from this study it appears that skin lesions were unlikely to be associated with the effects of eco-tourism, as the prevalence of skin lesions was higher in pool 9 where eco-tourism was largely land-based, in addition, the proportion of dolphins affected was not significantly different between pools 1 and all other pools, except for pool 9.

3. Geographical Isolation:

Pool 9 is a smaller pool (1 km^2) compared to pool 1 (2 km^2) (Beasley et al., 2014), and dolphin movement from this pool is restricted by waterfalls upstream and rapids downstream. As a result of these features the dolphins are largely confined together. This was confirmed photographically with the six adults and one juvenile in pool 9 always photographed together

and identified at every survey, demonstrating a close association between these animals. This close association may contribute towards the host factors in disease transmission, for example it may facilitate the transmission of infectious agents between individuals (Johnson et al., 2019), as well as appearing to maintain a constant endemic level within this small isolated group. This confinement may also expose these seven individuals to anthropogenic stressors from eco-tourism (Beasley et al., 2014; Dove, 2008), boat traffic, fishing pressures (Limsong et al., 2017) and anthropogenic contamination. Pool 9 has >40 villages surrounding it on both the Cambodian and the Laos PDR side compared to the 135+ households around pool 1 (Beasley et al., 2014). The heavier human population density around pool 9 is likely contributing to the higher prevalence of skin lesions in the dolphins in this pool.

6.4.2 Incidental findings

6.4.2.1 Age

This study presents the results from 99% of the marked (identified) MRID population (Ryan et al., 2011). Marks are naturally occurring in this population and are acquired over time. Calves are not born with markings (Ryan et al., 2011) and most, apparently unmarked animals, are of a young age. In addition, young calves (<1 year) appear to be more elusive and "boat shy" than older dolphins, and therefore more difficult to re-sight. Although insufficiently distinct for confident re-identification between surveys, subtle distinctions occur between all fins such that I was able to differentiate unmarked animals (previously not identified) within the same group/session during a survey. Given the small group sizes, with an average of 6 dolphins and a low proportion of unmarked animals, photo-identification was believed to be a reliable method for this study. In this study, very few juveniles were ever seen and photographed. This may be due to the low recruitment rate reported previously by

Ryan et al. (2011). Thus, the findings in this study predominantly pertain to adult animals, with the exception of one juvenile dolphin with tattoo lesions that was also subsequently submitted for necropsy examination on its death (Chapter 4 - Case 2).

6.4.2.2 Potential aetiology of the skin lesions

The aetiology of the skin lesions in this population has not yet been determined. In Chapter 4, the histological analysis undertaken by Kuiken (2010 pers. comm.) of one of the photo-identified dolphins from this study (Case 2) with "tattoo-like" skin lesions, detected an inflammatory reaction. This supported the belief that, in this animal, the lesions were an ante-mortem finding, and that it was unlikely the bacteria cultured were post-mortem invaders. In a study by Russo et al. (2018) it was demonstrated that the skin of dolphins (*T. truncatus*) supported a vast array of microorganisms, largely as a result of their habitat and proximity to anthropogenic activities. Dolphins in closer proximity to human populations were shown to have more microbial organisms, most likely associated with terrestrial urbanised runoff/effluent, than dolphins that did not have a close association with human populations. Whether the skin lesions observed in other MRIDs seen in the current study were also caused by bacterial agents remains to be investigated. Similarly, it is unknown whether or not there was a viral component to these lesions, as virology could not be undertaken in any of the studies undertaken as part of this research. Van Bressem et al. (2014) documented the first report of a cutaneous disorder in free-ranging, estuarine and riverine *O. brevirostris*. They reported the presence of cutaneous nodules in vulnerable populations of Irrawaddy dolphins from Malaysia, India (Chilika Lake) and Bangladesh. Comparable to that found in this study, they found the disease prevalence ranged from 2.2 to 9.1% in Malaysia (n=11-117) and was 13.9% (n=72) in Chilika Lake. In their study they also sampled skin lesions from one dead *O.*

brevirostris from Kuching and detected histological features consistent with fibropapillomas. However, as only one animal was sampled, the long-term impact of skin lesions on the viability of the affected dolphin populations still remains uncertain (Van Bressem et al., 2014). Further research on MRIDs needs to include a focus on the aetiology of the skin lesions; and in particular viral studies should be included in such research.

6.4.2.3 Skin lesions and contaminants

Van Bressem et al. (2014) believed that there may have been an association between the presence of skin disease in *O. brevirostris* and the levels of environmental contaminants present in Chilika Lake, with the progression and severity of skin disease being greatest in areas of high contaminant loads, in Kuching and Chilika Lake dolphins, compared to dolphins from other Malaysian locations and in Bangladesh. Environmental contaminants, such as persistent organic pollutants (POPs), can depress the immune system, increasing the risk of infectious diseases (Bennett et al., 2001; Hall et al., 2006; Ross, 2000), and this is believed to be a potential pathophysiological mechanism in the development of skin lesions in dolphins (Van Bressem et al., 2014).

6.4.2.4 Implications of skin lesions

The frequent finding of skin lesions in MRID, which has not previously been described in this population, is concerning. Disease can be a significant driver of species extinction (Epstein et al., 2016; Smith et al., 2006; Smith et al., 2009). Recently, disease has been linked to extinction risk in wildlife such as transmissible facial tumour disease in Tasmanian devils (*Sarcophilus harrisii*) (Epstein et al., 2016), white nose syndrome in bats (little brown bat - *Myotis lucifugus* and Indiana bat - *Myotis sodalis*) (Frick et al., 2010; Hoyt et al., 2016;

Thogmartin et al., 2013) and chytridiomycosis in multiple species of frogs (Skerratt et al., 2016; Wilber et al., 2017). In addition, there is now substantial evidence that diseases can greatly impact local species populations by causing temporary or permanent declines in abundance (Cunningham et al., 2021; Reeves et al., 2009; Smith et al., 2009; Smith et al., 2006; Wilber et al., 2017); however declines in dolphin abundance due to disease have not previously been reported in the literature, and further research should focus on the implications of skin lesions on this critically endangered population.

6.4.3 Study limitations

There were four main limitations with the study described in this chapter.

Firstly, the data collection in this study may underestimate the true prevalence of skin lesions within the MRID population. This is because photo-identification surveys are limited in that they can only be used to detect lesions on easily visible body parts, such as the dorsal fin and the dorsum immediately caudal and cranial to the dorsal fin (Hart et al., 2012). However, despite this limitation, photo-identification remains a cost-effective method for obtaining baseline skin lesion prevalence data in this population (Hart et al., 2012).

Secondly, this study largely reports on the prevalence of skin lesions in adult dolphins, as MRID calves were found to be notoriously shy and incredibly difficult to photograph, thus these data may underrepresent the true prevalence of skin lesions in this population, and as such the prevalence reported should be noted as a conservative value. Studies of skin lesions on cetaceans have indicated differential susceptibility and severity between different age groups (de Swart et al., 1994; Van Bressem and Van Waerebeek, 1996; Van Bressem et al.,

2009). However, age-class identification from photo-identification data is often limited to differentiating adults from calves, which may not provide useful information for diseases that commonly occur among juveniles, for example tattoo skin disease (de Swart et al., 1994; Van Bressem et al., 2009) as was observed in one case necropsied as part of the study reported in Chapter 4. Due to this limitation the prevalence of skin lesions in different age classes cannot be determined from photo-identification studies alone.

Thirdly, there may have been a bias in the photographs taken between the dry and wet season. In the dry season there was less water to cover, and so this facilitated finding the dolphins with less effort, resulting in more time to view and photograph the dolphins present – as opposed to the wet season when there was more river to transverse and so it was harder to find dolphins and to photograph them.

Finally, in this study, the photographic picture quality improved over time, as better equipment and improved lenses were used in the later surveys. In addition, improved photographer ability occurred with each survey effort. Thus, the data for the earlier surveys may further underestimate the true prevalence of skin lesions. As such it is recommended that future research continue to document the prevalence of skin lesions in this population using high quality cameras and lenses, to assess if there is an increasing or decreasing trend in the prevalence and location of the lesions.

6.5 Conclusions

The study presented in this chapter represents the first documented report of skin lesions occurring in a live population of MRIDs, and will serve as a baseline study for this population for future comparative studies. Whilst it remains uncertain as to the aetiology of the skin lesions, any disease condition found in a population on the verge of extirpation is of concern. As skin lesions can be a sensitive indicator of ill-health and immunosuppression, as well as environmental and anthropogenic impacts at a population level, and given that skin lesions were prevalent throughout the geographical range of the MRID, the next chapter will focus on environmental contaminants in dolphins from this population and their potential role in the health of this population.

CHAPTER SEVEN

Toxicological analysis of deceased Mekong River Irrawaddy dolphins from Cambodia

7.1 Introduction

In the previous chapters neck lesions and skin lesions were identified in MRIDs, and a hypothesis generated that immunosuppression may play a role in these conditions. A suggested aetiology for this immunosuppression included exposure to immuno-toxic environmental contaminants.

7.1.1 Contaminants in river dolphins

Very few published studies have examined environmental contaminants, such as heavy metals and POPs, in river dolphins (Senthilkumar et al., 1999). To date the only studies available are on Ganges River dolphins (*Platanista gangetica*) (Kannan et al., 1997; Kumari et al., 2002; Senthilkumar et al., 1999) and Irrawaddy dolphins (*O. brevirostris*) (Dove, 2009; Kannan et al., 2005; Murphy et al., 2009; Schnitzler et al., 2021). Unfortunately little is currently known about the toxicological status of MRID dolphins.

7.1.2 Mercury contamination in Cambodia

Heavy metal contamination in MRIDs has primarily focused on mercury (Hg) contamination. Mercury contamination in MRIDs has been investigated in two studies (Dove, 2008; Murphy et al., 2009) (Table 7-1). Since fish are the most likely source of Hg for dolphins, investigation of the concentration in fish has been undertaken (Murphy et al., 2009). In

addition, studies to assess the level of Hg exposure in humans living alongside the Mekong River were also conducted by Murphy et al. (2009).

Table 7-1: Mercury levels from studies carried out in Cambodia

Animal type	**Samples analysed or location site**	**n**	**Mean Hg level** (µg/g)	**Range** (µg/g)	**Reference**
MRIDs	Liver	4	1.25	0.86-1.54	Dove, 2009
	Liver	10	8.107	0.707-67.4	Murphy et al., 2009
	Kidney	4	0.3	0.28-0.31	Dove, 2009
	Blubber	4	0.053	0.03-0.07	Dove, 2009
	Brain	1	0.2		Dove, 2009
Fish (Mekong River)	Phnom Penh	15	0.043	<0.01-0.12	Agusa et al., 2005
	Kratie	9	0.158	0.015-0.171	Cheng et al., 2013
	Kampong Cham	9	0.025	0.023-0.027	Cheng et al., 2013
	Kandal	9	0.012	0.012-0.012	Cheng et al., 2013
	Tonle Srepok, Tonle Kong, Stung Treng, and Kratie	160	0.099	0.008-0.642	Murphy et al., 2009
	Kratie: Kampi Pool	31	0.12		Murphy et al., 2009
Humans (hair)	Phnom Penh, Kien Svay, Tomnup Rolork and Batrong,	94	3.1	0.54-190	Agusa et al., 2005
	Along the Mekong River from central to Northern Cambodia	78	3.89	23	Murphy et al., 2009
Environment	Sediment		<0.064		Murphy et al., 2009

Gold mining operations that use Hg to amalgamate the gold are found alongside the Mekong River, and Agusa et al. (2005) believed this was the primary source of Hg contamination

within the Mekong River, particularly in Kratie province (Cheng et al., 2013). Murphy et al. (2009) reported that one dolphin had substantially elevated levels of Hg compared with nine other carcasses tested, and suggested that this dolphin may have been feeding in close proximity to these gold mines.

7.1.3 Persistent organochlorine pollutants (POPs) in Cambodia

A preliminary study by Dove (2008; 2009) examined POPs in five stranded Irrawaddy dolphins in the Mekong River between 2004 and 2007. These results were compared to those found in a study by Kannan et al. (2005) who examined POPs in five stranded Irrawaddy dolphins in Chilika Lake, near Orissa, India, in 2000 and 2001. The average level of contaminants for the five Cambodian animals (4 calves, 1 juvenile) was higher than that in the five adult dolphins from Chilika Lake (e.g., ΣDDTs at 7800 vs. 5052ng/g lipid; ΣPCBs at 360 vs. 214ng/g lipid; ΣCHLDs at 56 vs. 21.7ng/g lipid; ΣPBDEs at 21 vs. 8.1ng/g lipid; HCB at 39 vs. 10.6ng/g lipid).

In Cambodia, there is limited information about the contamination level and distribution of POPs due to the lack of monitoring programs, not only in foodstuffs, but also in the environment (Wang et al., 2011). The Environmental Justice Foundation (EJF) reviewed the pesticide situation in Cambodia and found that inappropriate pesticide use, including their timing, frequency, concentration, and types of pesticides used, were widespread (EJF, 2002). These observations were also supported in the study of Krahn et al. (2007) who found evidence of the continued use of pesticides in Asia which precipitated the ‘Alaskan contaminant signature’, due to these pesticides being transported to Alaska via air and ocean currents, as well as by migratory fish stocks. High levels of DDT congeners, indicative of

recent use, were found in Alaskan ceteaceans due to the Alaskan signature (Krahn et al., 2007).

POPs, such as PCBs and DDT and their metabolites, have been used extensively in Cambodia for industrial purposes, agriculture, and vector control/public health programs (Kumar et al., 2013; Minh et al., 2006). Despite the World Health Organization (WHO) imposing a ban on the use of certain POPs in the 1970's, Cambodia and its neighbouring countries continue to use these compounds (Jensen et al., 2011; Kumar et al., 2013; Matsukawa et al., 2016; Schreinemachers et al., 2020). Koma et al. (2000) recorded 241 pesticides being used in Cambodia from October 1999 to June 2000, 19 of which were banned at that time by the WHO, including DDT and chlordane, and 71 were considered restricted for use but banned for importation into Cambodia. In 1992 The Stockholm Convention on Persistent Organic Pollutants (2001) officially banned the use of DDT for agricultural purposes in most Southeast Asian countries, including Cambodia (McCallum et al., 2009; Minh et al., 2006). Cambodia became a signatory to the Stockholm convention on the 23rd May 2001; however the country lacked the institutional capacity to effectively integrate these agreements into operational policies (Ramos-Sánchez, 2015). This was highlighted in the findings of the EJF (2002) and Jensen et al. (2011) who subsequently found that highly toxic pesticides belonging to WHO class I + II were still extensively being used in November 2006. For example, Sodavy et al. (2000) found that vegetable growers were significant users of pesticides, including highly neurotoxic agents (organophosphate, organochloride, and carbamate pesticides). These included DDT, Folidol® (methyl parathion), mevinphos®, Monitor® (methamidophos), Azodrin® (monocrotophos) and Furadan® (carbofuran). Chak

et al. (2010) also reported that the use of pesticides, such as DDT, was widespread, with farmers even in the smallest of villages in the most remote parts of the country using them.

In a study by Monrith et al. (1999) the concentrations of POPs, such as PCBs, DDTs, HCHs, CHLs, and HCB, were measured in 27 species of marine and freshwater fish collected in Cambodian waters. In their study, freshwater fish were found to have higher concentrations of DDTs, which highlighted the probable source as originating from agricultural runoff into inland watersheds, such as the Mekong River.

The current study was conducted on historical archived tissue samples from 22 dead MRIDs collected from the Mekong River between 2005 and 2010, to determine the level of immuno-toxic contaminants in this population. Understanding the levels of these contaminants is important to evaluate their contribution towards immunosuppression in the population and thus their impact on the health status of the MRIDs.

7.2 Materials and methods

7.2.1 Sample collection

In this study, MRIDs were necropsied according to the protocols previously described in Chapter 4. The protocols described for toxicology sample collection were adapted from Rowles et al. (2001) (Table 7-2). All samples collected from dolphins necropsied in Cambodia between 2005 and 2010 were shipped by the WWF-Cambodia to Büsum, Germany and subsequently analysed at the Research and Technology Centre Westcoast (FTZ), University of Kiel. Financial constraints restricted toxicological analyses to 22

animals. I formulated a priority list for the selection of these animals, based on the carcass score at necropsy (all CS2 or 3), microbiological investigations undertaken, and the presence or absence of neck lesions. One high priority dolphin, based on the freshness of the carcass, did not have internal organs available for heavy metal analysis, as these had been removed by the villagers prior to collecting this dolphin for necropsy. This dolphin was tested for POPs as it was freshly deceased, and an additional dolphin was selected for heavy metal analysis based on the priority list. Thus, in total, 22 dolphins were used in this study (Table 7-3).

Numerous studies have shown that age and sex have a significant effect on the residue levels of many compounds in tissues, thus demographic data were collected for an accurate interpretation of the tissue residue data (Rowles et al., 2001). Where available, the most likely cause of death ascertained at necropsy was included. The dolphins were assessed for evidence of neck lesions and/or fisheries interaction. A subset of the 22 dolphins in this study also had samples submitted for microbial culture and examination at the Pasteur Institute in Phnom Penh, and histological analysis as previously described in Chapters 4 and 5.

The tissues that were collected for the assessment of lipophilic, persistent and non-persistent organic pollutants were blubber (D, E, and F sample sites, Figure 7-1) and liver, and for heavy metals included kidney and liver. A minimum of 100g of full thickness blubber was collected from the lateral thorax, using a sterile stainless-steel knife or scalpel blade that had been soaked in 70% alcohol. During collection care was taken to ensure that tissues did not come into contact with intestinal contents or blood. To minimise contamination all tissues were rinsed in 70% alcohol prior to being stored. Three tissue samples were collected for each dolphin, and each placed in a pre-labelled food-grade plastic container, a plastic zip-lock bag, and wrapped in aluminium foil and stored at -20°C in Kratie Township, until the CITIES

permit (E-04406/09) was granted by the Cambodian Government to export the samples to the University of Kiel. The samples were transported for four hours on ice to the capital city Phnom Penh, where they were stored at -20˚C until transported to Germany. Samples were stored in specimen bags, placed in thermally insulated boxes with 15kgs of dry ice for the transportation to Germany.

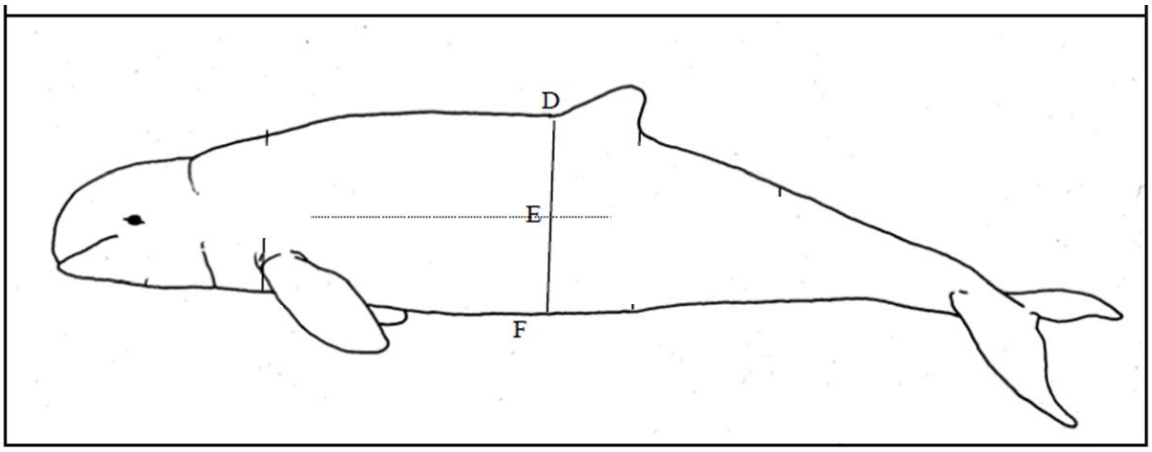


Figure 7-1 Sampled blubber areas.

7.2.2 Sample preparation

Frozen blubber samples from the 10 prioritised (Table 7-3) dolphins were homogenised. During the shipment of the samples, some labels and packaging were damaged, consequently these samples were not analysed. This resulted in some dolphins having insufficient quantities of fat from location D for analysis. In these cases, samples from sites D, E and F for individual dolphins were pooled together, homogenised and analysed. In one dolphin, samples D and E were lost in shipment, resulting in samples from site F only being analysed.

Samples of liver from two dolphins were also lost during shipment, resulting in POP results only being available for these animals. Consequently, two additional dolphins had heavy metals analysed, but no POP levels.

7.2.3 Dolphins in this study

The data for the 22 dolphins included in this study are summarised in Table 7-3. There were 20 dolphins analysed for POPs and 20 analysed for heavy metals, however results were only obtained from 18 of these. The pool in which the dead dolphin was found in, or if located outside of a pool, the closest pool upstream (allowing for currents to take dolphin carcasses downstream) was used as the dolphin source location.

Table 7-2: Protocols for specimen collection for chemical pollutants

Pollutants/heavy metals analysed for	Specimen	Collection site	CS	Amount	Storage
POPs: Organochlorine Pesticides Polychlorinated Biphenyls Brominated Flame Retardants Organotin Compounds Perfluorinated Tensides	Blubber	Blubber: Lateral thorax- full thickness from sites : D, E, F	2 & 3	100g optimal (Minimum 20g)	Blubber was frozen at -20˚C and stored in • a plastic container • a zip-lock bag • aluminium foil
	Liver	Left caudal lobe of liver			Liver was frozen at -20˚C and stored in • a plastic jar
Metals: Hg As Cd Cu Fe Pb Se Zn MeHg	Kidney	Left kidney	2 & 3	100g optimal (Minimum 20g)	Samples were frozen at -20˚C and stored in • a plastic container • a zip-lock bag • aluminium foil
	Liver	Left caudal lobe of liver			

POPs: Persistent organic pollutants, Hg: Mercury, As: Arsenic, Cd: Cadmium, Cu: Copper, Fe: Iron, Pb: Lead, Se: Selenium, Zn: Zinc, MeHg: Methyl-Mercury, CS: Carcass Score

Table 7-3: Summary data of dolphins sampled in this study

Age class	Case #	Specimen #	Length (cm)	Weight (kg)	Sex	CS	Pool #	NL	FI	Tissue sampled			Heavy metals	POPs
										Blubber*	Liver	Kidney		
Adults	1	CID 09 002	218	110	Female	2	1	-	-	D, E, F	+	+	+	+
	2	CID 09 005	200	80	Female	2	1	U	U	D, E, F	[redacted]			+
	3	CID 07 010	224	128	Female	3	3	+	-	D	+	+	+	+
	4	CID 07 011†	230	138	Female	3	1	+	-	D	+	+	[redacted]	+
	5	CID 08 005	220	106	Male	2	3	+	-	D, E	+	+	+	+
	6	CID 09 008	218	110	Male	2	1	-	-	F	+	+	+	+
	7	OBRE05-06/01	213	101.5	Male	2	6	U	+	D, E, F	+	+	+	+
	8	OBRE05-17/12	206	104.5	Male	2	2	U	+	D, E, F	+	+	+	+
Juveniles	21	CID 08 003	122	28	Female	2	1	+	-	D, E	+	+	+	+
	22	CID 09 001	137	42	Female	2	5	+	-	D, E	+	+	+	+
Calves	9	CID 07 002	95	9.8	Female	2	2	+	-	D	+	+	+	+
	10	CID 07 012	107	17	Female	2	1	+	-	D, E	+	+	+	+
	11	CID 07 014	69	3.8	Female	2	1	+	-	D, E, F	+	+	+	+

Age class	Case #	Specimen #	Length (cm)	Weight (kg)	Sex	CS	Pool #	NL	FI	Tissue sampled			Heavy metals	POPs
										Blubber*	Liver	Kidney		
	12	CID 08 001	104	14.5	Female	2	1	+	-	D, E	+	+	+	+
	13	OBRE06-09/12	109	12.5	Female	2	1	+	-	D	+	+	+	+
	14	OBRE05-16/12	106	15.2	Female	2	1	+	-	D, E, F	+	+	+	+
	15	CID 07 009	93	9	Male	2	6	+	-	D, E	+	+	+	+
	16	OBRE06-13/01	89	10.6	Male	2	1	+	-	D, E, F	+	+	+	+
	17	OBRE06-13/02B	107	14.6	Male	2	1	+	-	D, E, F	[redacted]			+
	18	CID 09 003	104	14.5	Male	3	3	+	-	D, E	+	+	+	+
	19	CID 07 013	104	13.2	Female	3	6	-	-	N/A	+	+	+	[redacted]
	20	CID 08 002†	101	13	Male	3	2	+	-	N/A	+	+	[redacted]	

*Sites sampled from Fig 7-1, CS= Carcass Score, N/A= not available, NL: neck lesion, FI: fisheries interaction, POPs: persistent organic pollutants, U: unknown, Black: no sample available, + presence of lesions or samples tested for specific substances; - absence of lesions, †Samples submitted to laboratory, but were not able to be analysed.

7.2.4 Heavy metals

The livers of 18 MRIDs were analysed for arsenic (As), lead (Pb), cadmium (Cd), iron (Fe), copper (Cu), total mercury (Hg), methylmercury (MeHg), selenium (Se) and zinc (Zn). Additionally, cadmium (Cd) was analysed from the kidney of these dolphins. The 18 dolphins comprised 6 adults, 2 juveniles and 10 calves.

Table 7-4: Summary morphometrics of the dolphins tested for heavy metals

Age class	**Mean length (m) (SD)**	**Mean weight (kg) (SD)**
Adults (n=6)	2.165 (0.06)	110 (9.4)
Juveniles (n=2)	1.3 (0.11)	35 (9.9)
Calves (n=10)	0.98 (0.12)	12.01 (3.84)

The heavy metal analyses were carried out by The Public Health Institute, Ostrava, Czech Republic (Zdravotní ústav se sídlem v Ostravě) and by GALAB Laboratories GmbH (Geesthacht, Germany). The Public Health Institute was chosen for their expertise in heavy metal analysis. Additional samples were subsequently analysed due to budget allowance at GALAB, resulting in testing of heavy metal content at the two facilities. At the Public Health Institute various protocols were used for the heavy metal analysis. At GALAB Laboratories the liver and kidney samples were analysed. The methods used for the heavy metal analysis, included AAS-CZL 2/95 for Hg and HPLC/ICP-MS-C ZL 2/08 for MeHg, and SOP OV 201.03 (ČSN EN ISO 17294-1, ČSN EN ISO 17294-2) was used for the rest of the elements analysed.

All data were expressed based on the fresh weight of the samples. The elemental concentrations in the tissues were reported in mg/kg. These results were directly converted to µg/g (ppm) for comparison with other published studies.

The molar ratio of Se:Hg was calculated by dividing the concentration (in µg/g) of the two heavy metals by their respective molecular weight (200.59 for Hg and 78.96 for Se). A mean Se:Hg molar ratio was calculated from the average Se and Hg levels (Burger and Gochfeld, 2013; Ralston et al., 2007).

7.2.5 Persistent organochlorine pollutants (POPs): organic compounds

Samples were prepared and analysed for POPs at GALAB Laboratories. The analysis of the POPs and PCB's were performed by Gas Chromatography-Negative Chemical Isolation-Mass Selective Detector (GC-NCI-MSD). The brominated flame retardants were measured by GC-MSD. The organotin compounds were analysed by GC with an Atomic Emission Detector (GC-AED). The perfluorinated tensides were analysed by Liquid Chromatography-Mass Spectrometry-MS (LC-MS-MS). The dioxins and furans were analysed using a GC/MS followed by analysis by high resolution gas chromatography.

The blubber from 20 individual dolphins were analysed for organic pollutants by GALAB Laboratories (Reports A2010101885 and A2010104227). The samples were analysed for brominated flame retardants (BFR), polychlorinated biphenyls (PCBs), organochlorine pesticides (OCP), dioxins and furans (PCDD/PCDF). Additional funding was obtained to test the blubber of five of these dolphins for organotin compounds (OTC), perfluorinated tensides (PFOs/PFOA), toxaphene and methyl parathion.

The gravimetric fat content was measured to generate a percentage fat content (ng/g) for each sample in order to assess the level of decomposition of each sample and for comparison of the results with other studies.

In this study, I summed together the congeners (Appendix 7). The concentration of DDE, p,p' was calculated for each dolphin, and summed together, to provide an overall proportion of the total ∑DDT. In this study, the terms DDT, DDE, and DDD are used to refer to the sum of isomer concentrations of: p,p'-DDT and o,p'-DDT; p,p'-DDE and o,p'-DDE; and p,p'-DDD and o,p'-DDD, respectively. DDTs refers to any or all of the six compounds listed above, as well as the metabolites and degradation products of these six compounds. ∑DDTs refers to the sum of the concentrations of p,p'-DDT, o,p'-DDT, p,p'-DDE, o,p'-DDE, p,p'-DDD, and o,p'-DDD. ∑PBDE refers to the sum total of all PBDE congeners analysed, ∑PCBs refers to the sum total of all PCB congeners analysed, and ∑CHLDs refers to the sum of the congeners chlordan-cis, chlordan-trans and nonachlor-trans. ∑Dioxin refers to the sum of all the dioxin congeners.

To express the overall toxicity of the dioxins the results were converted to "International Toxic Equivalents" (TEQ) (Van den Berg et al., 2006). Each compound was attributed a specific "Toxic Equivalency Factor" (TEF). This factor indicates the degree of toxicity compared to 2,3,7,8-TCDD, which is allocated a reference value of 1. The TEQ is operationally defined by the sum of the products of the concentration of each compound multiplied by its TEF value and is an estimate of the total 2,3,7,8-TCDD–like activity of the mixture (Van den Berg et al., 2006). This weights the toxicity of the less toxic compounds as fractions of the most toxic TCDD. To calculate the total TCDD TEQ of a dioxin mixture, the

amounts of each toxic compound were multiplied with their TEF and then summed. The TEF values published by Van Den Berg et al. (2006) were used in this study (Appendix 8) to convert the results calculated in the lipid into TEQ pg/g. As dioxins accumulate in fat, the burden was expressed in picogram I-TEQ per gram of serum lipid, (pg I-TEQ/g lipid). The TEQ was calculated for dioxins in this study so that the results could be easily compared with other studies.

POP results from this study were compared to the preliminary results for five MRIDs dolphins (four calves and one juvenile) from Dove (2008, 2009). Two of the four calves in this earlier study were re-analysed in this study (Cases 16 and 17). The results of one juvenile from Dove (2008) were pooled with the data in this study and compared to the results from Irrawaddy dolphins in Chilika Lake India (Kannan et al., 2005).

7.2.6 Statistical analyses

All contaminant data were assessed for normality using a Shapiro-Wilk test. All POPS, except for HCB data, were not normally distributed. In contrast, all data of heavy metals were normally distributed, except for Pb. Based on these results a Kruskal-Wallis ANOVA test was selected for the overall analysis of all of the contaminant data, with one measurement variable (either the heavy metal or the POP of interest), and one nominal variable (age class, sex, neck lesions, or fisheries interaction) selected for each analyses. When a significant difference was detected on the Kruskal-Wallis analysis, pairwise comparisons between groups were carried out using the post-hoc Dunn test, with a Šidák correction to adjust α for multiple comparisons. The data were analysed using the Real Statistics Resource Pack software (Release 5.4) (Zaiontz, 2018) for excel, and α was set at $p<0.05$.

7.3 Results

7.3.1 Heavy metals

The results of the heavy metal assays are summarised in Table 7-5.

7.3.1.1 Age

Overall there was a significant difference between the three age classes for Hg (H=7.46, d.f. 2; p=0.023), MeHg (H=8.9, d.f. 2; p=0.012) and Cu (H=13, d.f. 2; p=0.001). Using the post-hoc Dunn's test, the following heavy metals were significantly higher in the adult dolphins sampled than in the calves: Hg (11.21µg/g vs. 1.51µg/g, p=0.019); and MeHg (7.23µg/g vs. 0.72, p=0.033). In contrast, the calves had significantly higher levels of liver Cu (40.72µg/g) than adults (3.49µg/g) (p=0.0005). Adults also had a significantly higher level of Hg (11.21 µg/g vs. 0.91 µg/g, p=0.026) and MeHg (7.23 µg/g vs. 0.17 µg/g, p=0.006) than juveniles.

There were no results available for the juvenile age class for As, Cd (liver and kidney) and Pb. There was a significantly higher level of As and Pb from the liver and Cd from the kidney samples from adults than calves (As: H=7.5; d.f. 1; 0.13µg/g vs. 0.04µg/g; p=0.006; Pb: H=5.2; d.f. 1; 0.18 µg/g vs. 0.01; p=0.01; and kidney Cd: H=6; d.f. 1; 0.47µg/g vs. 0µg/g; p=0.014).

Overall there was no significant difference between the three age groups for Cd, Fe, Se and Zn from the liver (all $p \geq 0.13$).

Overall the mean molar Se:Hg ratio was significantly different between the age classes (H=10.95, d.f. 2; p=0.004) with the ratio significantly higher in calves (2.27, p=0.039) and juveniles (2.94, p=0.007) than in adults (0.95).

7.3.1.2 Geographical location

In this study, the dolphins sampled originated from pools 1 (n=10), 2 or 3 (n=6), 5 (n=1) and 6 (n=3). Overall there was no significant difference found in the concentration of any of the heavy metals between dolphins from different pools (all p-values ≥0.38) and consequently no further comparisons were made for the pool data.

7.3.1.3 Neck lesions

The level of Zn in the liver of adults with neck lesions (28.7µg/g) was significantly lower (H=3.85, d.f. 1, 2; p=0.049) than that of adults without evidence of neck lesions (65.17µg/g). There were no other significant differences in the levels of heavy metals between these two groups - Hg (p=0.27); As (p=0.5); Cd (liver p=0.5); Cd (kidney; p=0.12); Cu (p=0.8); Pb (p=0.56); Se (p=0.5); and MeHg (p=0.8). There was also no significant difference found in the Se:Hg ratios in adults with and without neck lesions (p=0.4), and also in calves with and without neck lesions (p=0.6).

Table 7-5: Results of heavy metal analysis (in µg/g)

Age class	Case #	Hg	As	Cd	Cu	Fe	Pb	Se	Zn	MeHg	Cd	Se:Hg (Molar)
Adults	1	9.24	0.088	0.4	1.77	887	0.056	2.26	18.9	6.5	13.8	0.62
	3	18.4	0.17	1.0	3.5	717	0.79	6.9	31.3	3.12	-	0.95
	5	16.0	0.067	0.3	4.08	488	0.015	4.92	35.9	15.0	2.69	0.78
	6	10.8	0.082	0.7	3.84	331	0.016	3.65	54.4	6.86	2.3	0.86
	7	11.8	0.242	0.3	6.03	551	0.024	5.63	59.6	11.5	0.1	1.21
	8	1.0	-	0	1.7	60	-	0.49	81.5	0.42	-	1.24
	Samples (n)	6	5	6	6	6	5	6	6	6	4	6
	Mean	11.21	0.13	0.47	3.49	505.72	0.18	3.98	46.93	7.23	4.72	0.91
	Median	11.30	0.09	0.39	3.67	519.50	0.02	4.29	45.15	6.68	2.50	0.91
	Standard deviation	6.05	0.07	0.34	1.62	290.28	0.34	2.34	22.64	5.34	6.16	0.25
	95%CI lower	3.13	0.05	0.33	0.34	181.73	0.64	1.57	6.69	5.42	10.43	0.46
	95%CI upper	17.56	0.22	0.82	5.18	810.34	0.60	6.43	70.69	12.84	14.52	1.43

Age class	Case #	Hg	As	Cd	Cu	Fe	Pb	Se	Zn	MeHg	Cd	Se:Hg (Molar)
Juveniles	21	0.41	-	-	4.0	247	-	0.54	88.9	0.12	-	3.35
	22	1.4	-	-	6.3	237	-	1.4	48.0	0.22	-	2.54
	Samples (n)	2	0	0	2	2	0	2	2	2	0	2
	Mean	0.91	-	-	5.15	242.00	-	0.97	68.45	0.17	-	2.94
	Median	0.91	-	-	5.15	242.00	-	0.97	68.45	0.17	-	2.94
	Standard deviation	0.70	-	-	1.63	7.07	-	0.61	28.92	0.07	-	0.57
	95%CI lower	1.37	-	-	3.19	13.86	-	1.19	56.68	0.14	-	1.83
	95%CI upper	7.19	-	-	19.76	305.53	-	6.43	328.29	0.79	-	4.06

Age class	Case #	Hg	As	Cd	Cu	Fe	Pb	Se	Zn	MeHg	Cd	Se:Hg (Molar)
Calves	9	2.09	0.034	<0.003	18.6	458	0.007	1.02	76.8	1.82	0.02	1.24
	10	0.67	0.025	<0.003	7.27	354	0.015	0.54	93.7	0.6	<0.003	2.05
	11	2.13	0.058	<0.003	40.2	210	0.009	1.54	28.5	-	0.01	1.84
	12	1.07	0.058	0	35.8	463	0.018	1.0	44.7	0.68	0.01	2.37
	13	1.7	0.032	<0.003	19.9	526	0.007	0.9	110	1.02	0.01	1.34
	14	0.93	-	-	36.3	671	-	1.1	42.0	0.22	-	3.00
	15	1.07	0.026	<0.003	17.8	294	0.01	0.78	29.6	0.88	0	1.85
	16	1.5	-	-	86.4	179	-	3.4	18.6	0.47	-	5.76
	18	0.96	-	-	31.9	307	-	0.55	77.3	0.19	-	1.46
	19	3.0	-	-	113	236	-	2.1	74.7	0.61	-	1.78
	Samples (n)	10	6	6	10	10	6	10	10	9	6	10
	Mean	1.51	0.04	<0.003	40.72	369.80	0.01	1.29	59.59	0.72	0.01	1.84
	Median	1.29	0.03	<0.003	33.85	330.50	0.01	1.01	59.70	0.61	0.01	1.84
	Standard deviation	0.72	0.02	-	33.33	156.83	0.00	0.88	30.95	0.49	0.01	1.33
	95%CI lower	0.99	0.02	-	16.87	257.61	0.01	0.67	37.45	0.34	0	0
	95%CI upper	2.03	0.05	-	64.56	481.99	0.02	1.92	81.73	1.10	0.02	4.88

Age class	Case #	Hg	As	Cd	Cu	Fe	Pb	Se	Zn	MeHg	Cd	Se:Hg (Molar)
P-value	Overall‡	0.023*	0.006*#	0.134	0.0015*	0.24	0.01*#	0.94	0.6	0.012*	0.014*#	0.004*
	Adults vs. calves	0.019*	0.006*#	N/A	0.0005*	N/A	0.01*#	N/A	N/A	0.033*	0.014*#	0.039*
	Adults vs. juveniles	0.026*	-	N/A	0.54	N/A	-	N/A	N/A	0.006*	-	0.007*
	Juveniles vs. calves	0.44	-	N/A	0.09	N/A	-	N/A	N/A	0.15	-	0.3

-No result obtained from the laboratory analysis, * $p<0.05$, ‡Overall p-value from the Kruskal-Wallis test for all groups containing tested individuals, N/A: post hoc Dunn test not applicable as overall Kruskal-Wallis test was not significant. #data only available for adults and calves.

7.3.2 Persistent organochlorine pollutants (POPs)

In this study, concentrations of organochlorines in dolphin blubber decreased in the order of DDTs > PCBs > CHLs > HCB > PBDEs > Dioxins > OC (Table 7-6).

7.3.2.1 Age

The levels of dioxins were significantly different between the three age classes in pg/g (H=12.18, d.f. 1, 2; p=0.002) and in TEQ (H=11.7, d.f. 2; p=0.003). The level of ∑Dioxins were significantly higher in adults (34.47pg/g) than in calves (2.91pg/g, p=0.007). The TEQ levels of ∑Dioxins were also significantly higher in adults (2.27TEQ) than in calves (1.15TEQ, p=0.004), and significantly higher in juveniles (3.57TEQ) than in calves (p=0.0094).

Overall there was a significant difference in the levels of PCBs across the three age classes (H=9.37, d.f. 2, p=0.009). The level of PCBs in adults (346.9ng/g) was significantly higher (p=0.019) than in calves (142.75ng/g), and also significantly higher in juveniles (705.4ng/g) than in calves (p=0.012). The other POPs were not found to be statistically different between the three age classes - ∑PBDE (p=0.22), ∑CHLD (p=0.5), ∑DDT (p=0.26), ∑HCB (p=0.98), and OC (p=0.56).

7.3.2.2 Sex

Overall there was a significant difference between male and female dolphins for dioxins (pg/g) (H=13.76, d.f. 4, p=0.008); dioxins TEQ (H=12.41, d.f. 4, p=0.01); and PCBs (H=11.29, d.f. 4, p=0.02). However, there was no significant difference between the sexes for

levels of ∑PBDE (p=0.37), ∑CHLD (p=0.786), ∑DDT (p=0.259), HCB (p=0.867) and ∑OC (p=0.37).

Adult females had higher ∑Dioxin levels (61.3pg/g; 2.27TEQ) than female (3.7pg/g, p=0.0017; 1.15 TEQ p=0.02) and male calves (2.8pg/g, p=0.003; 0.99 TEQ, p=0.01). The female juveniles in this study also showed significantly higher ∑Dioxins (3.57TEQ) than female (1.15 TEQ, p=0.02) and male calves (0.99 TEQ, p=0.01). Adult males had significantly higher PCB levels (606.8ng/g) than female (163.2ng/g, p=0.01) and male calves (142.8 ng/g, p=0.03). In addition, the two female juveniles in this study were found to have significantly higher levels of ∑PCBs (705.4ng/g) than both female (163.2ng/g, p=0.016) and male calves (142.8ng/g, p=0.03).

7.3.2.3 Geographical location

In this study, the 20 dead dolphins analysed for POPs were associated with pools 1 (n=12), 2 or 3 (n=5), 5 (n=1) and 6 (n=2). Overall there was no significant difference in the level of any POPs and the originating pool - ∑PBDE (p=0.19), ∑CHLD (p=0.45), ∑DDT (p=0.23), HCB (p=0.39), ∑Dioxin (p=0.14), ∑PCB (p=0.2) and ∑OC (p=0.67). The highest concentration of ∑DDT was found in an adult male (180,088ng/g lipid) from pool 6. Of the five animals with the highest levels of DDT, 80% (n=4) were from pools 1-3. A level of 32,274ng/g was found in the smallest calf in the study (Case 11) from pool 1.

7.3.2.4 Neck lesions

There was no significant difference between the overall levels of POPs in the eight dolphins affected by neck lesions, compared to the two dolphins with no evidence of neck lesions -

∑PCBC (p=0.86), ∑CHLD (p=0.527), ∑DDT (p=0.88), ∑HCB (p=0.67), OC (p=1), ∑Dioxins (p=0.179), and PCBs (p=0.427).

7.3.2.4.1 POPs in two different Irrawaddy dolphin populations

The levels of POPs reported in MRIDs in this study and Dove (2008) were compared to the levels in Chilika Lake Irrawaddy dolphins (Kannan et al., 2005). The levels of ∑PBDE (37.5ng/g), ∑CHLDs (89.3 ng/g), ∑DDT (12,000ng/g) and ∑HCB (40.9ng/g) were all significantly higher in the MRIDs than in the Chilika Lake Irrawaddy dolphins (∑PBDE 2.5ng/g, p=0.0027; ∑CHLDs 3.2ng/g, p=0.0036; ∑DDT 2,000ng/g, p=0.032; and ∑HCB 5.3ng/g, p=0.0007). In contrast, there was no statistical difference between the two groups for ∑PCBs (MRIDs 299.7ng/g vs. Chilika Lake 71ng/g, p=0.2).

Table 7-6: Results from POP analysis (ng/g Lipid), and Dioxins (pg/g and pg I-TEQ/g)

Age Class	Sex	Case #	Length (m)	Weight (kg)	∑PBDE ng/g Lipid	∑CHLD ng/g Lipid	∑DDT ng/g Lipid	∑HCB ng/g Lipid	∑Dioxin pg/g	∑Dioxin pg I-TEQ/g	∑PCB ng/g Lipid	∑OC ng/g Lipid
Adults	Females	1			18.3	-	3497	32.1	60.1	2.52	232.6	18.7
		2			103.7	51.4	9689	59.9	30.9	2.02	299.7	-
		3			64.7	121.5	55470	19.4	73.6	4.68	340.1	51.9
		4			46.5	5973.0	1534	-	62.5	2.00	-	-
		Mean			58.30	2048.63	17548	37.13	56.78	2.81	290.80	35.30
	Males	5			24.7	89.5	18854	38.0	16.3	1.33	353.7	-
		6			90.0	169.1	43897	70.2	24.9	1.60	606.8	-
		7			155.0	488.4	180088	49.5	38.0	2.65	889.1	-
		8			208.8	-	23874	-	21.8	3.78	-	-
		Mean			119.63	249.00	66678	52.57	25.25	2.34	616.53	
		n	8	8	8	6	8	6	8	8	6	2
		Mean	2.16	109.75	88.96	1148.82	42113	44.85	41.01	2.57	453.7	35.3
		Median	2.18	108	77.35	145.3	21364	43.75	34.47	2.27	346.9	35.3
		SD	0.10	17.43	65.96	2368.57	58896	18.70	21.51	1.14	248.40	23.48
		95%CI lower	2.08	95.18	33.82	0	0	25.23	23.03	192.98	0	2.08
		95%CI upper	2.24	124.32	144.11	3634.48	91351	64.47	59.00	714.35	246.22	2.24
		Minimum	2	80	18.3	51.4	1534	19.40	16.28	1.33	232.60	18.70
		Maximum	2.3	138	208.8	5973	180088	70.20	73.60	4.68	889.10	51.90

Age Class	Sex	Case #	Length (m)	Weight (kg)	∑PBDE ng/g Lipid	∑CHLD ng/g Lipid	∑DDT ng/g Lipid	∑HCB ng/g Lipid	∑Dioxin pg/g	∑Dioxin pg I-TEQ/g	∑PCB ng/g Lipid	∑OC ng/g Lipid
Juveniles	Females	20			0.8	80.6	33049	84.0	24.3	3.19	412.5	-
		21			160.3	744.4	151297	23.9	33.9	3.94	998.3	-
		Mean			80.55	412.50	92173	53.95	29.10	3.57	705.40	
		n	2	2	2	2	2	2	2	2	2	-
		Mean	1.30	35.00	80.55	412.50	92173	53.95	29.10	3.57	705.40	-
		Median	1.30	35.00	80.55	412.50	92173	53.95	29.10	3.57	705.40	-
		SD	0.11	9.90	112.78	469.38	83614	42.50	6.79	0.53	414.22	-
		95%CI lower	0.34	0	0	0	0	0	0	0	0.34	-
		95%CI upper	2.25	123.94	1093.87	4629.69	843414	435.77	90.09	4427.05	2.25	-
		Minimum	1.22	28.00	0.80	80.60	33049	23.90	24.30	3.19	412.50	-
		Maximum	1.37	42.00	160.30	744.40	151297	84.00	33.90	3.94	998.30	-
Calves	Females	9			35.3	86.2	14164	54.8	9.3	1.87	348.2	-
		10			-	-	1317	-	2.9	0.71	43.8	-
		11			24.1	60.5	30275	39.1	5.4	1.25	255.2	-
		12			12.6	-	3121	26.5	2.8	0.70	45.2	-
		13			-	-	1005	-	4.5	1.05	76.6	5.2
		14			39.7	237.4	80586	40.9	2.5	1.78	249.8	-
		Mean			27.93	128.03	21745	40.33	4.57	1.23	169.80	5.20

Age Class	Sex	Case #	Length (m)	Weight (kg)	∑PBDE ng/g Lipid	∑CHLD ng/g Lipid	∑DDT ng/g Lipid	∑HCB ng/g Lipid	∑Dioxin pg/g	∑Dioxin pg I-TEQ/g	∑PCB ng/g Lipid	∑OC ng/g Lipid
		15			20.1	-	8028	55.0	0.9	0.63	335.6	7.7
		16			58.8	25.1	6833	36.1	2.6	1.34	86.3	120.7
	Males	17			21.7	-	35613	-	2.9	0.00	94.0	-
		18			70.4	134.8	37767	-	30.5	1.35	191.5	-
		Mean			42.75	79.95	22060	45.55	9.23	0.83	176.85	64.20
		n	10	10	8	5	10	6	10	10	10	3
		Mean	0.98	12.15	35.34	108.80	21871	42.07	6.44	1.07	172.62	44.53
		Median	1.04	13.50	29.70	86.20	11096	40.00	2.91	1.15	142.75	7.70
		SD	0.124	3.91	20.21	82.25	25047	11.11	8.76	0.57	118.42	65.97
		95%CI lower	0.74	4.49	18.44	6.67	3954	30.40	0.17	87.91	0	0
		95%CI upper	1.23	19.81	52.24	210.93	39789	53.73	12.70	257.33	208.42	29.54
		Minimum	0.69	3.80	12.60	25.10	1005	26.50	0.93	0.00	43.80	5.20
		Maximum	1.09	17.00	70.40	237.40	80586	55.00	30.50	1.87	348.20	120.7
P-value	Overall‡				0.221	0.503	0.261	0.98	0.002*	0.028*	0.009*	0.563#
	Adults vs. calves				N/A	N/A	N/A	N/A	0.0007*	0.004*	0.019*	0.563
	Adults vs. juveniles				N/A	N/A	N/A	N/A	0.808	0.077	0.87	-
	Juveniles vs. calves				N/A	N/A	N/A	N/A	0.039*	0.015*	0.021*	-

-no laboratory result available, *significant $p<0.05$. ‡Overall p-value from Kruskal-Wallis test for all groups containing tested individuals. N/A: post hoc Dunn test not applicable as overall Kruskal-Wallis test was not significant. # data only available for adults and calves.

7.4 Discussion

7.4.1 Key findings

This study represents the largest toxicological study carried out to date on MRIDs. There were three important findings in this study: namely POPS were significantly higher in the sampled MRIDs than reported in the study of Kannan et al. (2005) in Indian Irrawaddy dolphins; immuno-toxic heavy metals were found to be present in the sampled dolphins; and lower levels of zinc were associated with the presence of neck lesions in the sampled adult dolphins. These findings are discussed below.

7.4.1.1 POPs in the Mekong River Irrawaddy dolphin population

The nutritional status and body condition of an animal can affect the measured tissue concentrations of POPs (Debier et al., 2006). As such, I examined the gravimetric lipid percentages, which indicated that all the samples were fresh, with minimal sample degradation and the lipid contents were similar to those reported by Kannan et al. (2005) in Irrawaddy dolphins from Chilika Lake, India. The concentration of HCBs ($p=0.0007$), ∑CHLDs ($p=0.0037$), ∑DDT $p=0.032$ and ∑PBDEs ($p=0.0027$) were all significantly higher in the Cambodian MRIDs than in the Chilika Lake *O. brevirostris*. According to Kannan et al. (2005), the actual cause of mortality in Chilika Lake dolphins were mostly attributable to small boat propeller strikes, whereas the current research would indicate that the cause of death in the MRIDs examined are largely associated with infectious disease in calves, similar to the condition necrotising fasciitis of humans, and fisheries interactions with gillnets in adults. The significantly higher levels of POPs in the MRID may also be contributing to ill-health in this population, similar to that described by Taylor et al. (2021b) with POPs from

maternal transfer to the neonatal population of Australian sea lions (*Neophoca cinerea*) and Australian fur seals (*Arctocephalus pusillus doriferus*).

∑DDT and its metabolites were the predominant contaminants found in the MRIDs, in particular DDE-p,p' was the predominant congener in this study, accounting for 90% of the ∑DDT. Kannan et al. (2005) found that DDE-p,p' accounted for 77% of the total DDT in the Chilika Lake Irrawaddy dolphins. The higher proportion of DDE-p,p' in the MRIDs suggests exposure to aged residues from the environment as DDT breaks down to DDE-p,p' and DDD-p,p', two relatively stable metabolites (Kannan et al., 2005).

The levels of PCBs in the MRIDs were not significantly different from that found in Chilika Lake dolphins (333.4ng/g and 176.8ng/g, respectively, p=0.197). This is not surprising given that both Chilika Lake and the Mekong River are bordered by agricultural land rather than urban and industrial land (Kannan et al., 2005), resulting in lower environmental contamination with PCBs. However, levels of PCBs are influenced by age and sex (Wells et al., 2005) and hence comparisons of values in different populations need to consider differences in the demographics of these populations.

7.4.1.2 Heavy metals in the Mekong River Irrawaddy dolphin population

In the current study, heavy metals, in particular Hg, As, Cd, and Pb, were found in the sampled dolphins. These four heavy metals have no known function in animals, and are considered toxic if found in any detectable concentration in animals, including dolphins (Dórea 2019; Siebert and Das, 2010). The detection of Hg in this study is of concern, given its known immuno-toxicity in dolphins at levels above 1µg/g (Béland et al., 1991; Colborn

and Smolen 2003; Pellisso et al., 2008), as is the detection of As due to its toxicity and reported association with skin lesions in humans from Cambodia (Kubota et al., 2006; Phan et al., 2014) and elsewhere (Chen et al., 2009).

7.4.1.3 Mercury

The results in this study suggest that the MRIDs are exposed to levels of Hg that can result in immunosuppression. Pellisso et al. (2008) reported reduced B and T cell mitogenesis resulting in immunosuppression in bottlenose dolphins (*T. truncatus*) when Hg levels were >11µg/g, and at these elevated concentrations cellular apoptosis can occur. Similarly, Béland et al. (1991) found that concentrations of Hg in beluga whales (*D. leucas*) above 2µg/g, significantly suppressed proliferation of splenocytes and thymocytes *in vitro*. In the MRIDs in this study, the concentrations of Hg were in excess of 1µg/g in 78% (95%CI 52, 94) of the dolphins tested and it is likely these results are biologically significant, affecting the health of the population. These immuno-toxic levels of Hg were reported in all adults (n=6), 70% of the calves (n=10) and 50% (one) of the juveniles (n=2) sampled in this study, and the concentration was significantly higher in adults (11.21µg/g) than in calves (1.51µg/g). Murphy et al. (2009) previously reported similar results from MRIDs, with 90% (n=10) of the dolphins tested having Hg levels >1µg/g, with one dolphin having a Hg level of 67.4µg/g. Thus, the levels of Hg found in the MRIDs in the current study, indicate that the dolphins would likely have a reduced lympho-proliferation response with reduced B and T cell mitogenesis, which can render the dolphins susceptible to both bacterial and viral infections (Shen and Fillatreau, 2015; Zhao et al., 2018). This is likely because B-cells in dolphins produce IgG that bind with pathogens and toxins, and has an important role in antibody-dependent cell-mediated cytotoxicity. Furthermore, T–cells, such as natural killer (NK) cells,

play an important role in innate immunity by lysing and killing non-self/foreign cells, tumour cells and virus-infected cells (Fair et al., 2017). The immune system of dolphins appears to be more sensitive to Hg exposure than that of humans (Colborn and Smolen, 2003), and the reduction of any functional activity of the MRIDs immune system may reduce their resistance to disease (Pellisso et al., 2008, Bennett et al., 2001).

In this study, the highly toxic MeHg was found in higher concentrations in adult MRIDs, with levels reaching 15µg/g, compared to calves (1.82µg/g) and juveniles (0.22µg/g). The reason for the lower levels in the juveniles is not clear, however may reflect different exposure, such as trans-placental and lactational transfer for neonates, and dietary exposure in juveniles. However, only two juveniles were sampled in this study and further juveniles need to be sampled to provide confidence in these results. Mollenhauer et al. (2009) found that MeHg disrupts normal cellular homeostasis, as well as altering gene expression, reducing cellular translation and proliferation in *T. truncatus*. Mercury present in fish is exclusively MeHg (Carey and Bryant, 1995; Rosas and Lehti, 1996), with fish in the Mekong River containing up to 0.96µg/g of MeHg (Murphy et al., 2009). Ronald et al. (1977) fed harp seals (*Pagophilus groenlandicus*) fish containing 0.25µg/g MeHg, and the seals subsequently developed anorexia, weight loss and lethargy within 2-3 months, with blood parameters indicating toxic hepatitis, uraemia and renal failure. Although the level in fish being consumed by the dolphins in the Mekong River is less than that reported to induce mortality in seals (25µg/g) (Ronald et al., 1977), it is in excess of that reported to have caused ill-effects in *P. groenlandicus* (0.25µg/g), and thus this finding is of concern in the MRID population that is declining and suffering from the effects of ill-health.

The Hg results in adults in this study were higher than those reported in dead juvenile beluga whales (*D. leucas*), aged 0-3.5 years (range 0.07-1.84µg/g, n=5) (Bernier et al., 1996), however, were lower than a number of oceanic dolphin species (Bilandžić et al., 2012). In this latter study of Bilandžić et al. (2012), some stranded Risso's dolphins (*G. griseus*) were found to have Hg in excess of 1000µg/g in the liver, more than 5-times higher than the levels in bottlenose (*Tursiops* spp.) and striped dolphins (*S. coeruleoalba*). This highlights the complexities involved in simply reporting a Hg value in dolphins, as is commonly done in the literature, as the range across cetaceans is wide and varied.

7.4.1.3.1 Mercury and selenium

In the current study, the mean Se:Hg molar ratios were significantly different between the three age classes, being >1 in calves and juveniles (2.27; 2.94, respectively) and <1 in adults (0.95). Ralston (2008) suggested that Se:Hg molar ratios >1 were probably protective for adverse Hg effects. However, interpretation of the molar ratio of Se to Hg is controversial (Bennett et al., 2001; Brockman et al., 2011; Ralston et al., 2007; Ralston et al., 2016; Ralston and Raymond, 2010). For example, Bellante et al. (2017) found that the molar ratio was influenced by age, being <1 in adult striped dolphins (*S. coeruleoalba*), and >1 in calves and juveniles, similar to that found in this study in the MRIDs. Furthermore, Burger et al. (2012) reported that the ratio assumes complete bioavailability of Hg and Se, and does not account for interactions with other unspecified substances. Based on the molar ratio in this study, toxic effects of Hg may be occurring in the adult MRIDs; however, in calves and juveniles there was a two-fold excess of Se compared to Hg. High levels of both Hg and Se are immuno-toxic, making the host more susceptible to infectious diseases. For example, in a study by Bennett et al. (2001) they found higher mean liver concentrations of Hg and Se in

harbour porpoises (*P. phocoena*) that died of infectious disease, compared with presumably healthy porpoises that died from physical trauma. However, overall the Se concentrations in the MRID tissues sampled in this study were lower than that found by Bryan et al. (2007) in *T. truncatus*. The difference appears to be consistent with that observed in ocean versus freshwater fish (Burger and Gochfeld, 2013), with Ralston and Raymond (2010) finding hazardous Se:Hg molar ratios in freshwater fish. Thus, the MRIDs, which consume freshwater fish, may be more vulnerable to the toxic effects of MeHg than their oceanic counterparts.

7.4.1.4 Arsenic

The As concentrations in dolphins from the Mekong River were lower than those found in humans in Cambodia (Gault et al., 2008; Kubota et al., 2006), who had levels exceeding those associated with As toxicity (1µg/g) described by Parsons and Chan (1998). In humans, skin lesions are the earliest clinical sign of chronic As exposure, and Kubota et al. (2006) reported an elevated prevalence of skin lesions in the Kratie region. It is possible that chronic As exposure could also pose a threat to the MRIDs, as was seen with the prevalence of skin lesions, including pool 1 (Kratie region), in Chapter 6. In this study, As levels were only recorded in dead dolphins from pools 1-3 and 6, thus further research is required to determine if As is playing a role in the presence of skin lesions in the MRID population.

7.4.1.5 Neck lesions and contaminants

In this study, the only significant finding associated with adult dolphins with neck lesions was a significantly lower level of Zn ($p=0.049$) compared to adults without neck lesions. There was only one calf without a neck lesion and consequently there was no difference

between the levels in calves with and without neck lesions. In this study, although calves had a higher mean level of Zn (59.59 µg/g) than adults (46.93 µg/g), this difference was not significant. Further research is required to ascertain if the association between Zn and neck lesions observed in adults is also extrapolatable to calves.

7.4.2 Incidental findings

7.4.2.1 Age

Firstly, there was no significant difference between POP levels in the adults and calves for all contaminants studied except for dioxins and PCBs, which were significantly higher in the adults. Additionally, there was no difference found in the levels of POPs in the adult males and the two juvenile females. This is because both male and female dolphins accumulate POPs rapidly as juveniles (de Moura et al., 2008). In males, the accumulation continues throughout life, whereas in females the level can eventually decrease due to placental (Desforges et al., 2012; Greig et al., 2007; Wade et al., 1997) and lactational transfer (Béland, 1996; de Moura et al., 2008; Ridgway and Reddy, 1995; Rosas and Lehti, 1996; Wade et al., 1997; Wells et al., 2005). The transfer of contaminants to foetuses and neonates is not unique to dolphins, and has also been well documented in humans (Al-Saleh et al., 2012; Bergonzi et al., 2011; Katić et al., 2010; Pohl and Tylenda, 2000; Qu et al., 2010; Tan et al., 2009). Thus, as a result of this trans-placental and lactational transfer, MRID calves are starting their lives with levels of organic pollutants comparable to those of the adult male population.

7.4.2.2 Sex

Secondly, the only significant difference in the level of POPs in males and females, were found with PCBs and dioxins. The levels of PCBs were found to be significantly higher in males than in females. Older males have higher concentrations of POPs than younger males due to bioaccumulation with time (Wade et al., 1997). In contrast, Jepson et al. (1999) found that female dolphins had significantly lower levels of PCBs than males due to maternal transfer of POPs to offspring. Thus, the findings in this study indicate, in part, maternal transfer of PCBs to calves resulting in a reduced contaminant load in the adult females. This is discussed in more detail in the following section. However, in contrast to PCBs, females in this study had significantly higher levels of dioxins than males. The reason for this difference is unknown, and further research is required on these contaminants.

7.4.2.3 Maternal transfer

Thirdly, female dolphins are able to transfer their toxic contaminant load onto their calves via placental transfer *in utero* and via lactation. Both placental and lactational transfer lower the concentration of POPs in adult female dolphins, thus these animals exhibit a decrease in body levels with age (Wade et al., 1997). As such, the continued reproductive success of the older females lowers the concentrations of contaminants compared to younger females (Wade et al., 1997) and menopausal or reproductive senescence females.

7.4.2.3.1 *In utero* transfer

In this study, the POP levels in the smallest pre-term calf (Case 11) had the second highest ∑DDT levels, and had levels of PCBs which were in the higher range of ∑PCBs found in the calves. In killer whales (*O. orca*), calves have been shown to carry higher blubber

contaminant levels than adults (Krahn et al., 2007), as the transfer of maternal contaminant loads can be significant, especially for first born calves (Aguilar and Borrell, 1994; Borrell et al., 1996; de Moura et al., 2008). For example, Wade et al. (1997) and Desforges et al. (2012) found that near term beluga whale (*D. leucas*) foetuses, had around 10% higher tissue concentration of all POPs compared to their mothers. The results in Case 11 implicate placental transfer of DDT, PCBs, PCBDs, CHLDs, HCBs, and dioxins *in utero*. Gardner et al. (2007) reported similar findings in *P. phocoena*, with high POP levels in foetal samples, implicating gestational transfer from the mother to the calf. The findings in this study represent the first results supporting the likely *in utero* transfer of POPs in Irrawaddy dolphins.

Case 11 also had immuno-toxic levels of Hg (2.13µg/g) recorded, also indicative of *in utero* transfer. *In utero* transfer of Hg has been previously documented in Amazon River dolphins, (*I. geoffrensis*) (Rosas and Lehti, 1996) and in people (Schober et al., 2003). It is highly likely that exposure to Hg *in utero* is having a detrimental effect on the MRID calves. *In utero* exposure to Hg in humans can result in permanent neurological disabilities to the developing foetus, which is particularly sensitive to neuro-toxic and immuno-toxic heavy metals (Dórea, 2019; Nuttall, 2004).

7.4.2.3.2 Lactational transfer

Nursing dolphin calves can have extremely high concentrations of POPs (Béland, 1996; de Moura et al., 2008; Gauthier et al., 1998; Wade et al., 1997) due to the large quantities of lipophilic POPs potentially being ingested in the milk (Law, 1996; Ridgway and Reddy,

1995; Rosas and Lehti, 1996). Béland (1996) found that, in *D. leucas*, 70% of the mother's PCB load was transferred to the offspring via milk fat, so that within a year a beluga calf could attain twice the level of that found in the mother. Milk from dolphins has a very high fat content, with Eichelberger et al. (1940) reporting 108-180g/L of fat in milk from four dolphins (*T. truncatus*), compared to 6-60g/L in human milk (Corvaglia et al., 2008). POPS, such as dioxins, are highly lipophilic and therefore can be found in high levels in dolphin milk (Roeder et al., 1998). However, the milk fat content of Irrawaddy dolphins is unknown and further research is required to determine this. In the current study, it was impossible to ascertain which MRID calves were from which adult female, or if the mothers of calves were primiparous or multiparous. These can have a dramatic impact on the levels of individuals, consequently the paternity of mother-calf pairs is examined genetically in the following chapter.

Methylmercury was not detected in Case 11, which was in contrast to the findings in the other nine calves, which were presumed to be neonates based on their morphometrics. These nine calves had levels of MeHg ranging from 0.19-1.82µg/g, supporting the hypothesis that they had been suckled by contaminated mothers. The findings in Case 11 suggest that MeHg is initially transferred only through lactation in MRIDs, and subsequently through the consumption of contaminated fish. This transfer of Hg via lactation has also been documented by Frodello et al. (2002) and Rosas and Lehti (2002) in Amazon River dolphins (*I. geoffrensis*). Rosas and Lehti (1996) reported a concentration of 0.176µg/g in the milk of *I. geoffrensis*, which they claimed was close to the minimum level of MeHg toxicity (0.2µg/g) in non-pregnant human adults. Subsequently, Rosas and Lehti (2002) established that the total mercury in dolphin' milk is in the form of toxic MeHg, and milk is in osmotic equilibrium with blood (Rosas and Lehti, 1996). The current study did not examine the

concentration of Hg in milk, but only in the liver, with results 10 fold higher than those reported by Rosas and Lehti (1996) in the milk.

In this study, calves were found to have significantly higher levels of Cu compared to adults. The significance of this is unknown, however it may indicate defects in copper homeostasis, or reflect high Cu levels found in mammalian colostrum (Puchkova et al., 2018). Acute infections can also cause an increase in Cu (de Romaña et al., 2011; Milanino et al., 1993) with elevated levels of Cu reported in human neonates with early onset congenital infections (Wisniewska et al., 2017). Thus, further research is required to see if there is an association between elevated Cu levels in MRID calves and disease, such as necrotising fasciitis that appears to have a higher predilection for calves in this population.

7.4.2.4 PCBs

Finally, the mean PCB levels in adults (453.76ng/g) and calves (172.62ng/g) in this study were far lower than that reported in various other species of oceanic dolphins (Jepson et al. 2016). Additionally, the levels of PCBs in the MRID calves (142.75ng/g) were significantly lower to that found in a study by Gardner et al. (2007) in *P. phocoena* (4975 ng/g, p=0.01).

Although the levels of PCBs were lower for MRIDs than oceanic dolphins, the PCB 138 congener was the most abundant in this study. Brousseau et al. (2003) found that, of all the POPs detected in marine mammals, PCB congener 138 was the most immuno-toxic. Similarly, in a study by Yordy et al. (2010a) they found that, when PCB 138 and 180 were combined in a mixture, it induced a significant anti-oestrogenic response. In the current study of the dolphins sampled, 70% (14/20) had detectable levels of PCB 138 and 180 in their

blubber. Similar results have been reported in immunosuppressed belugas (*D. leucas*) (Muir et al., 1990). Thus, it is possible that the levels of congeners 138 and 180 found in this study may adversely affect the immune status of the MRIDs, particularly if other immuno-toxic compounds are already having an adverse effect, as discussed by Yordy et al. (2010a) who described the additivity nature of immuno-toxic compounds. Furthermore, due to the anti-oestrogenic effect of these contaminants, fertility in the female MRIDs could be impacted, as has been demonstrated in humans (Yang et al., 2008). This could contribute to the low recruitment in this population, which is examined further in the following chapter examining the genetic relatedness of the sampled MRIDs.

Several authors have suggested that there is an associative relationship between exposure of cetaceans to PCB's and mortality due to infectious disease (Bennett et al., 2001; Buschmann et al., 2007; Sodavy et al., 2000; Suzuki et al., 2006) as PCBs have been linked to deleterious effects on immune function, in addition to other effects on reproduction and endocrine homeostasis (Schwake et al., 2002). Jepson et al. (2005) found that PCBs in harbour porpoises were associated with infectious disease mortality at concentrations above 17,000ng/g, which is well in excess of that found in MRID in the current study. However, it may be that the particular PCB congeners, rather than the overall concentration are affecting the MRIDs, and further research is required to verify this.

7.4.3 Implications of exposure to contaminant in the MRIDs

In this study, a number of recognised immuno-toxic contaminants were detected in the sampled MRIDS, particularly POPs (DDT and PCBs) and heavy metals (Hg, Se, Cd and As). POPs are capable of altering both innate and acquired immune functions (de Guise et al.,

2003), and as such have been implicated in compromised immune functions in cetaceans from waters surrounding Hong Kong, resulting in high neonatal mortality rates (Jefferson et al., 2006, Law, 1996). In dolphins and other marine mammals, the suppression of immune function following exposure to POPs and heavy metals is well documented (Bernier et al., 1996; Brousseau et al., 2003; de Guise et al., 2003; de Swart et al., 1994; Jepson et al., 2005; Nakata et al., 2002; Van Loveren et al., 2000). Several studies have demonstrated causal associations through immuno-toxicology linking contaminants with an adverse effect on the immune system. For example, Lahvis et al. (1995) reported an association between the levels of PCBs and DDTs and immune suppression in free-ranging bottlenose dolphins (*T. truncatus*) and Borrell et al. (1996) found that striped dolphins (*S. coeruleoalba*) with higher burdens of POPs were more likely to die from a viral infection, such as morbillivirus, than those with lower levels.

Certain POPs have been reported to cross the placental barrier and affect foetal cells, resulting in impaired immune function and foetal toxicity, due to their endocrine-disruptive properties (Carvan III and Busbee, 2003; de Guise et al., 2003). Furthermore, peri-natal transfer of POPs through lactation can similarly affect the highly susceptible developing immune system of the calves (de Guise et al., 2003). However, the consequences of *in utero* placental transfer and post-natal transfer via lactation of POPs has only received attention since the late 1990's (Colborn and Smolen, 2003), and the long-term consequences are difficult to predict (Mössner and Ballschmiter, 1997). The implications of the maternal transfer of immuno-toxic contaminants to the MRID calves is not known, however, given the mortality of calves associated with neck lesions, and the marginal recruitment in this population, the precautionary principle should be invoked.

7.4.3.1 Implications of this study

In light of the findings in this study, there is an urgent need for environmental intervention to reduce exposure to contaminants that are now known to be accumulating in the MRID population. For example, Siebert and Das (2010) noted that the high percentage of organic Hg in this cetacean population was unusual. POPs were found to be significantly higher in MRID dolphins than in the Chilika Lake Irrawaddy dolphins, thus the levels of DDT and congeners, together with elevated Hg and highly toxic MeHg, are of concern for this population. It is likely that the exposure of the MRIDs to these immuno-toxic contaminants poses a threat to this population by causing immune dysfunction, making them more susceptible to opportunistic infections and thereby increasing the risk of mortality. This, coupled with the dolphins' longevity, increases the risk of harmful long-term chronic effects.

7.4.4 Limitations

There were several limitations to the current study.

Firstly, the costs associated with complete toxicological studies prohibited further samples from being analysed, despite a much larger collection of archived samples being available for analysis. When further funding becomes available these samples should also be analysed to expand the confidence in the toxicological results.

Secondly, although the findings in this study suggest that the levels of Hg and POPs are sufficient to be associated with mortality from infectious diseases, the inter-relationships between tissue concentrations of POPs, Hg and Se, as well as age, nutritional status and disease, are complex, thus caution should be taken in interpreting the results without further supporting evidence.

Thirdly, the samples in this study were predominantly from pools 1-3, and 6. This did not permit analysis between pools 1 and 9, which would have been a useful comparison, given the results reported in Chapter 6. In addition, the results from the different pools should be treated with caution as dead dolphins can be carried downstream and end up in a different geographical location to where they originated from.

Finally, there was only one calf without a neck lesion, and limited heavy metal testing and no testing for POPs was undertaken in this calf, severely restricting the comparison between calves with and without neck lesions. Thus, future studies should focus on analysing archived toxicological samples from dolphins (calves) without neck lesions to increase the sample size in this data set.

7.5 Conclusions

Immuno-toxic environmental contamination could be affecting the health status of the MRIDs. Contaminants, such as DDTs, Hg, and MeHg, were found in these dolphins in concentrations sufficient to cause immuno-toxicity, which can result singularly or synergistically in immunosuppression. In addition, immuno-toxic PCB congeners (138 and 180) may also synergistically be contributing to immunosuppression in the MRIDs. Thus, the bio-accumulation of these immunosuppressive contaminants may pose a threat to the health and viability of this cetacean population. In addition, the high concentration of maternal transfer of POPs to neonates, either during gestation or via lactation, as indicated in this study where neonatal calves (<110cm in length) had equivalent toxic loads to adults, may represent a greater immuno-toxic threat than exposure acquired as a juvenile or as an adult (Ross et al.,

1996), rendering neonates even more susceptible to infections and hence mortality, particularly as they rely predominantly on maternally acquired immunity.

In the following chapter the genetic variability of a subset of the MRIDs is reported to assess the genetic diversity in this population. In addition, paternity studies were conducted to assess if genetic factors may also be contributing to the population decline.

CHAPTER EIGHT

Multilocus analyses of the Mekong River Irrawaddy dolphins (*Orcaella brevirostris*) from Cambodia: insights into a recent population size decline

8.1 Introduction

In the previous five chapters, I examined environmental influences that may be directly or indirectly associated with mortality, such as environmental pollution, fisheries-interaction, disease and pathogens. In addition, I identified physiological host-factors likely associated with immunosuppression that may also be contributing towards mortality in this population. In this chapter, I further investigate physiological host-factors and their potential association with disease, by examining the population genetics of the MRIDs.

8.1.1 Genetics and extinction risk

Genetic factors may be involved in the loss of genetic diversity and increased extinction risk of wild populations (Frankham, 2005; Frankham and Ralls, 1998). This has been shown in the King island platypus (*Ornithorhynchus anatinus*) (Furlan et al., 2012) and in Pacific salmon (*Oncorhynchus* spp.) (Gustafson et al., 2007). However, in small populations, assessing the consequences of any loss of genetic diversity is often overlooked in favour of identifying and addressing the direct causes of mortality (Woodroffe et al., 2007), even though analysis of a population's genetic diversity is a valuable method of investigating a population's fitness. Different molecular markers, including sequencing of variable regions of the mitochondrial genome, analyses of nuclear neutral markers, such as microsatellite loci, and more recently analyses of full genomes (Hohenlohe et al., 2021), can all be used to

measure current population genetic diversity, as well as to understand the populations' trends and past population history (Baker et al., 1998; Dalebout et al., 2006; Gallego-García et al., 2018; Kretzmann et al., 2006; Satizabal et al., 2012; Wiemann et al., 2010).

The relationship between genetic variation and fitness has been widely studied by evolutionary and conservation biologists (Baker et al., 2006; Brüniche-Olsen et al., 2018; Edmands, 2007; Frankham, 2002; Frankham, 2005; Frankham, 2015; Fredrickson, 2007; Fredrickson et al., 2007; Houde, 2010; Kretzmann et al., 2006; Lucy et al., 2008; O'Brien and Evermann, 1988; O'Brien et al., 1986; Reed and Frankham, 2003; Shafer et al., 2015; Samuel and Bruno, 2002; Zschokke and Baur, 2002). The long term effects of diversity loss can affect both population health and reproduction, which in turn affects the potential survival of a species (Leigh et al., 2012). Diversity loss can then result in a small population size, which may result in inbreeding and further loss of genetic variation (Frankham and Ralls, 1998; Frankham et al., 2019). However, quantifying the extent to which inbreeding contributes to the extinction or extirpation of wild populations is extremely difficult to demonstrate (Frankham and Ralls, 1998). The following flow diagram (Figure 8-1) depicts the relationship between genetic factors that can result in increasing the extinction/extirpation risk in a population.

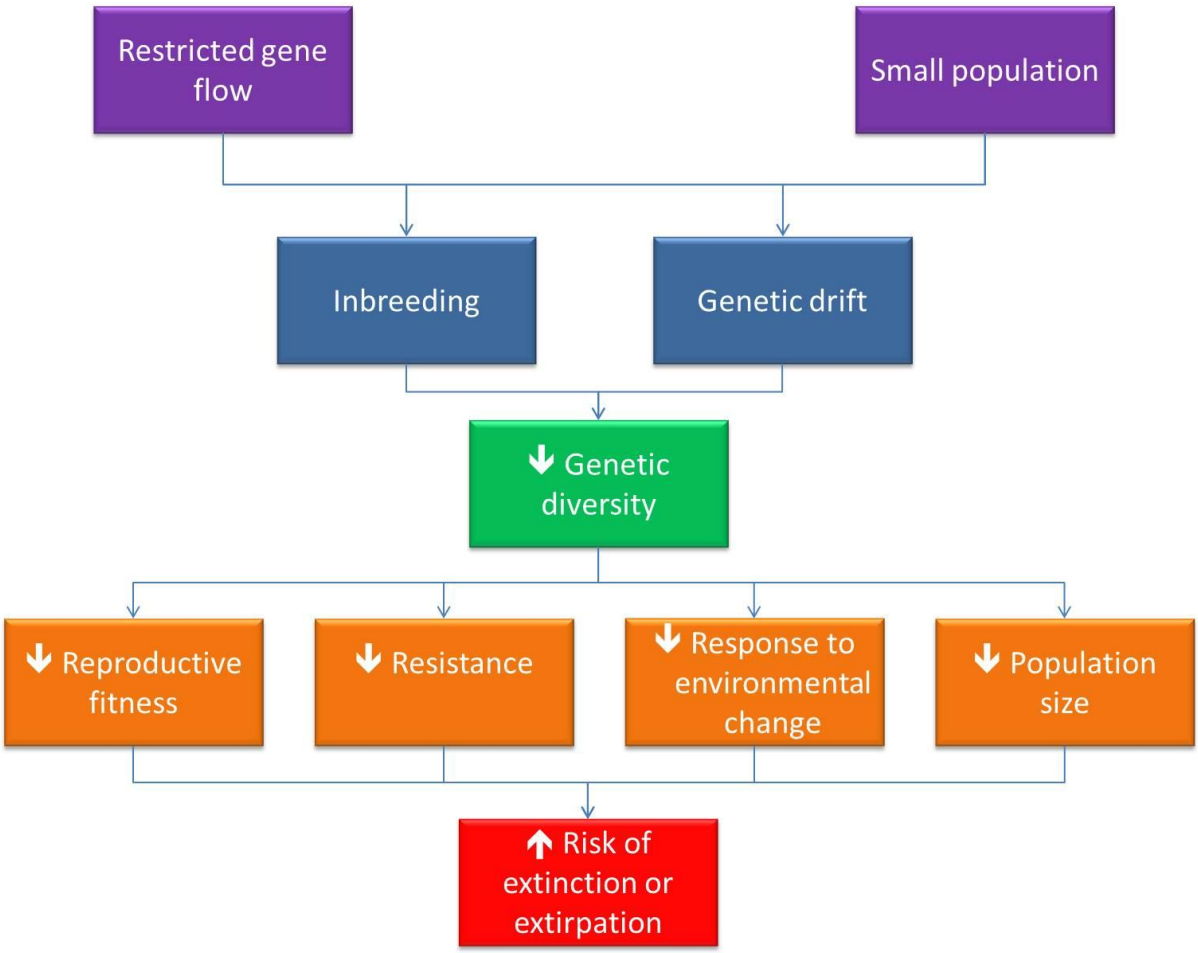


Figure 8-1: Flow diagram depicting genetic factors that can result in increasing the extirpation or total extinction risk in a population.

8.1.2 Genetics of the Mekong River Irrawaddy dolphins

Previously genetic comparisons among *Orcaella* spp. have only been reported by Beasley et al. (2005) and Jayasankar et al. (2011). Six mitochondrial DNA (mtDNA) Control Region (CR) haplotypes have been identified in *O. brevirostris* originating from Asia (Beasley et al., 2005), supporting the presence of two main clusters: one including the MRIDs in Cambodia, and the other representing the dolphins in the marine and freshwater habitats of Thailand, Indonesia and the Philippines. Geographical barriers that may affect the dispersal and gene flow of populations have possibly led to higher levels of population structure due to the limited genetic exchange between populations (Hollatz et al., 2011). For example, Caballero

et al. (2010) and Caballero et al. (2018b) demonstrated this in their studies in the Guiana dolphin (*Sotalia guianensis*) from different geographical regions, and in the tucuxi dolphin (*Sotalia fluviatilis*) also from different geographical regions within the Amazon basin. Little was known about the genetic diversity and population structure of the MRIDs, prior to the commencement of this study. However, through the analyses of genetic samples collected as part of this thesis, further genetic studies have subsequently been carried out. For example, a study by Krützen et al. (2018) used genetic samples from the current study to investigate population structure and demography by examining a 384bp control region for mitochondrial DNA and 21 microsatellites. They found that only 48% (n=10) of the microsatellite loci tested were polymorphic, with very low levels of genetic diversity detected in the population. Furthermore, in their study they identified that the current population size was only 5.2% of the ancestral population, with the decline occuring fairly recently in geological terms (around 145,000 years ago; 95%CI 159-1,584,893). Using the population estimate from the study reported by Ryan et al. (2011), this would suggest an ancestral population of approximately 1,650 MRIDs. The results of Krützen et al. (2018) indicate a long-standing isolation of the MRID population from other Irrawaddy dolphin populations, with extremely low nucleotide diversity and a lack of genetic structure of mitochondrial DNA in this population.

In this chapter, I present results of a detailed study of the MRID population structure and its genetic diversity, using analyses of a 647bp of mitochondrial DNA Control Region (mtDNA CR) and 12 neutral nuclear microsatellite loci. Firstly, I used the mtDNA CR to elucidate the phylogeographic relationship between the MRIDs and other *O. brevirostris* populations in Asia in order to understand the population structure at a macrogeographical scale (between geographical locations), and at a microgeographical scale (between groups within the Mekong River). Secondly, I used microsatellite loci to identify: a) levels of population

structure and gene flow among dolphins from the local pools in the Mekong River (Figure 6-1); b) parentage analyses and c) if the loss of calves and juveniles could be related to low genetic diversity levels. Finally, in an effort to understand the levels of neutral genetic diversity found across the range of *Orcaella* spp., I initiated collaboration with other Irrawaddy dolphin researchers who were studying these species across their range. That collaboration resulted in the study reported by Caballero et al. (2018a), the results of which will be discussed later in this chapter.

8.2 Materials and methods

8.2.1 Sample collection

A total of 44 skin samples from dead MRIDs from Cambodia were analysed in this study, of which DNA results were successfully obtained for 35 dolphins (Table 8-1). This number (n=35) represents 41% (95%CI 30.6, 52.4) of the population of 85 estimated by Ryan et al. (2011) in 2010. These samples were collected between 2004 and 2010 as described in Chapter 4 by three principal investigators (I. Beasley, M. Gilbert and V. Dove) from The Mekong Dolphin Conservation Project (MDCP), Wildlife Conservation Society (WCS), and The Cambodian Mekong Dolphin Conservation Project (CMDCP), respectively. All the samples were collected in conjunction with the Fisheries Administration of Cambodia (MAFF). The samples were either frozen at -20°C or stored in 70% alcohol or DMSO. Samples were imported to Colombia under CITES permit No. KH0669 and E-04406/09. The samples were sent to the Laboratorio de Ecología Molecular de Vertebrados Acuáticos (LEMVA), Universidad de los Andes in Bogota, Colombia, where I carried out all the genetic analysis under the supervision of Professor Susana Caballero.

Table 8-1: Summary data from samples collected

Case #	Specimen number	ID #	Pool*	Age	CS	Sex	D-Loop	Microsats	D-*loop* & microsats	Histopath	Toxicology	Neck lesion
1	OBRE06-13/01	53	9	Calf	2	Male	+	+	+	×	×	No
2	OBRE05-16/12	39	6	Adult	2	Male	+	+	+	×	✓	Unknown
3	OBRE04-28/09	23	6	Calf	2	Male	-	+	-	✓	✓	Yes
4	OBRE05-17/12	40	6	Calf	4	Female	+	+	+	×	×	Yes
5	OBRE05-06/01	41	6	Calf	3	Female	+	+	+	×	✓	No
6	CID 08 002	86	7	Calf	4	Male	-	+	-	×	×	Yes
7	CID 07 014	85	7	Calf	3	Male	+	+	+	×	×	Yes
8	CID 09 002	92	1	Calf	2	Female	+	+	+	×	✓	Yes
9	CID 09 005	93	1	Calf	2	Female	-	+	-	✓	✓	Yes
10	CID 08 004	87	1	Adult	2	Female	+	+	+	✓	✓	No
11	CID 08 001	88	1	Adult	2	Female	+	+	+	×	✓	Unknown
12	CID 09 001	94	1	Calf	2	Female	+	+	+	×	✓	Yes
13	CID 09 003	95	1	Adult	2	Male	+	+	+	✓	✓	No
14	CID 09 008	96	1	Adult	3	Female	+	+	+	×	✓	Yes
15	CID 07 009	80	1	Juvenile	3	Female	+	+	+	×	✓	Yes

Case #	Specimen number	ID #	Pool*	Age	CS	Sex	D-Loop	Microsats	D-*loop* & microsats	Histopath	Toxicology	Neck lesion
16	CID 07 003	74	1	Calf	2	Female	-	+	-	✓	✓	Yes
17	CID 07 004	75	1	Calf	3	Male	+	+	+	×	×	CNBD
18	CID 07 011	82	1	Calf	4	Female	+	+	+	×	×	Yes
19	CID 08 003	89	1	Calf	2	Male	+	+	+	×	✓	Yes
20	CID 07 012	83	2	Calf	3	Male	-	+	-	✓	✓	Yes
21	CID 07 007	78	2	Calf	4	Female	+	+	+	×	×	CNBD
22	CID 07 013	84	2	Calf	3	Female	+	+	+	×	×	Yes
23	CID 08 005	90	2	Calf	3	Female	+	+	+	×	×	Yes
24	CID 07 010	81	2	Calf	4	Male	+	+	+	×	×	CNBD
25	OBRE06-03/01	54	2	Calf	4	Male	+	+	+	×	×	CNBD
26	OBRE06-14/01A	55	3	Calf	3	Male	+	+	+	✓	✓	Yes
27	OBRE06-14/01B	56	3	Adult	2	Male	+	+	+	✓	✓	Yes
28	OBRE06-01/02	57	3	Adult	3	Female	+	+	+	×	✓	Yes
29	OBRE06-03/02	58	3	Calf	4	Female	+	+	+	×	×	Yes
30	OBRE06-13/02A	59	4	Calf	5	Unknown	+	+	+	×	×	CNBD
31	OBRE06-13/02B	60	4	Calf	4	Male	-	+	-	×	×	CNBD
32	OBRE06-15/02B	61	4	Calf	4	Female	-	+	-	×	×	CNBD

Case #	Specimen number	ID #	Pool*	Age	CS	Sex	D-Loop	Microsats	D-*loop* & microsats	Histopath	Toxicology	Neck lesion
33	ORBE06-28/02	62	1	Calf	2	Male	+	-	-	×	✓	Yes
34	OBRE05-20/01	42	1	Calf	3	Female	+	-	-	×	×	Yes
35	OBRE05-01/03	43	3	Calf	4	Female	+	-	-	×	×	CNBD
36	ORBE05-09/03	44	1	Calf	2	Female	-	-	-	×	×	No
37	ORBE05-18/03	45	2	Adult	4	Female	-	-	-	×	×	CNBD
38	ORBE05-19/03	46	2	Calf	4	Female	-	-	-	×	×	CNBD
39	OBRE05-24/05	47	6	Adult	4	Female	-	-	-	×	×	CNBD
40	OBRE05-10/12	48	6	Calf	4	Male	-	-	-	×	×	CNBD
41	OBRE05-25/01	49	2	Adult	2	Male	-	-	-	×	✓	CNBD
42	OBRE04-09/11	24	6	Calf	4	Male	-	-	-	×	×	Yes
43	OBRE04-10/11	25	1	Adult	3	Female	-	-	-	×	×	Yes
44	OBRE02-08/09	4	3	Juvenile	3	Female	-	-	-	✓	✓	Yes

*As displayed in Figure 6-1. ID # = identification number, CS=carcass score as defined in Chapter 4, Microsats= Microsatellites, Histopath= histopathology + results obtained, - results not obtained, ✓ Tested, × Not tested. CNBD = Could not be determined.

8.2.2 DNA Extraction, and mtDNA control region (CR) amplification and sequencing

Total DNA was extracted using the Qiagen DNeasy kit (Qiagen DNeasy, Valencia, CA, USA) following the manufacturer's guidelines. An approximate 700 base-pair (bp) region of the mtDNA CR was amplified using the previously published primers M13Dlp1.5 (5'-TGTAAAACGGCCAGTTCACCCAAAGCTGRARTTCTA-3') and (Reverse) Heavy-strand Dlp8 (5´- CCATCGWGATGTCTTATTTAAGRGGAA-3´) from Baker et al. (1998) and Caballero et al. (2007) following the amplification conditions in those studies. The PCR mix consisted of 32µl which included 19.6µL H_2O, 3µL reaction buffer (PCR buffer), 2µL bovine serum albumin (BSA), 3µL 1.5 mM $MgCl_2$, 0.3µL 200µM dNTPs, 1µL 0.4µM of primer 1, 1µL 0.4µM of primer 2 and 0.1µL 1U PlatTaq DNA polymerase (ThermoFisher), plus 2µL of DNA for each sample.

The polymerase chain reaction (PCR) cycling profile for the MtDNA sequencing consisted of two minutes of initial denaturation at 94˚C, followed by 34 cycles at 94˚C for 30 sec, annealing at 55˚C for 45 sec, extension at 72˚C for 40 sec, and a final extension at 72˚C for 10 minutes. PCR amplifications were performed using biolase taq (BIOTAQ®) DNA polymerase (Bioline Australia Pty. Ltd.). The PCR amplification was evaluated in a 3% agarose gel. Successful PCR products were cleaned using 10% polyethyleneglycol (PEG) and sequenced at Macrogen (Seoul, South Korea) using the standard protocols of BigDye™ on an ABI 3100 Perkin-Elmer automated capillary sequencer. All sequences were aligned using the sequencing software Geneious Pro version 4.8.5 (Drummond et al., 2009).

8.2.3 Microsatellite amplification and genotypification

All 35 samples were tested with a panel of 12 loci (Table 8-2). Of these, nine polymorphic loci were successfully amplified (GATA 98, EV94, AAT 44, PHO 142, MK6, SQUI003, SGUI006, SGUI016, and SGUI017). Forward primers were fluorescently labelled. The amplification conditions were as follows:

GATA98, EV94, AAT44: 2 min of initial denaturation at 94˚C, followed by 30 sec at 92˚C, 45 sec at 55˚C, 50 sec at 72˚C, repeating the last 3 steps for 15 cycles of denaturation, followed by 30 sec at 89˚C, 45 sec at 55˚C, 50 sec at 72˚C, repeating the last 3 steps for 20 cycles of denaturation, followed by 20 min at 72˚C, and then stored at 4˚C.

PPho142: 2 min of initial denaturation at 93˚C, followed by 30 sec at 92˚C, 45 sec at 60˚C, 50 sec at 72˚C, repeating the last 3 steps for 15 cycles of denaturation, followed by 30 sec at 89˚C, 45 sec at 60˚C, 50 sec at 72˚C, repeating the last 3 steps for 20 cycles of denaturation, followed by 20 min at 72˚C, and then stored at 4˚C.

SGUI 003, 006, 011, 016, 017, 018: 1 min of initial denaturation at 95˚C, followed by 30 sec at 95˚C, 45 sec at 57˚C, 45 sec at 72˚C, repeating the last 3 steps for 5 cycles of denaturation, followed by 30 sec at 92˚C, 45 sec at 57˚C, 55 sec at 72˚C, repeating the last 3 steps for 25 cycles of denaturation, followed by 20 min at 72˚C, and then then stored at 4˚C.

MK6 and MK9: 2 min of initial denaturation at 93 ˚C, followed by 30 sec at 92 ˚C, 45 sec at 50˚C, 50 sec at 72˚C, repeating the last 3 steps for 15 cycles of denaturation, followed by 30 sec at 89˚C, 45 sec at 50˚C, 50 sec at 72˚C, repeating the last 3 steps for 20 cycles of denaturation, followed by 20 min at 72˚C, and then stored at 4˚C.

The fragment run and the analysis of the nine microsatellite loci successfully amplified was performed on an Applied Biosystems ABI 3100 Genetic Analyser using the sizing ladder ROX500 (ThermoFisher).

Table 8-2: Microsatellite DNA primers for the 12 loci analysed

LOCUS	**Repeat motif**	Label	**Primer 5´-3´**	**Size range (bp)**	**GenBank accession #**	**Reference**
MK6	$(GT)_{17}$	NED	F-GTCCTCTTTCCAGGTGTAGCC R-GCCCACTAAGTATGTTGCAGC	145-189	AF237891	Krützen et al., 2001
MK9	$(CA)_{17}$	FAM	F-CATAACAAAGTGGGATGACTCC R-TTATCCTGTTGGCTGCAGTG	168-180	AF237893	Krützen et al., 2001
EV94	(TC)n (AC)n	FAM	F-ATCGTATTGGTCCTTTTCTGC R-AATAGATAGTGATGATGATTCACAC	198-261		Valsecchi and Amos, 1996
GATA98	(GATA)n	NED	F-TGTACCCTGGATGGATAGATT R-TCACCTTATTTTGTCTGTCTG	92-134		Palsboll et al., 1997
PPHO142	$(CA)_{22}$	VIC	F-GAAGGCTCAGGGTATTG R-CAGTTACTTTCCTCGGG	127-161	AF151789-1	Rosel et al., 1999
SGUI003	$(GT)_{28}$	FAM	F: TCCAATCTCCAACCAAATCCC R: GTCGCTAAGTTCATCATCTGC	148–162	BV693807	Cunha and Watts, 2007
SGUI006	$(GT)_{21}$	VIC	F: CTATGATGGACGGTTGAAGG R: TCTCTTGGTCATTGCCTTCC	201–215	BV693810	Cunha and Watts, 2007
SGUI011	$(GT)_{26}$	FAM	F: ACAGAGAAGCAAGTGGGAAACC R: TTCCCCGCCACTAAGATTCC	398–446	BV693813	Cunha and Watts, 2007
SGUI016	$(GT)_{28}$	NED	F: TTCTCTGGGCAAACACTGC R: CATTATTGCCGAACTGATGC	158–162	BV693815	Cunha and Watts, 2007
SGUI017	$(CA)_{22}$	VIC	F: GTGGTGGAGTAGAGGATAGG R: ACATTGGGCTTCAACGCACG	150–166	BV693816	Cunha and Watts, 2007
SGUI018	$(GT)_{29}$	FAM	F: CTGGAAAAAGAGTAGTTGGC R: GTGCAAGACCTCAAAATCC	234–252	BV693817	Cunha and Watts, 2007
TtruAAT44	$(AAT)_{12}$	FAM	U 5´-CCTGCTCTTCATCCCTCACTAA L 5´-CGAAGCCCAAACAAGTCATAGA	92	AF416501	Caldwell et al., 2002

8.2.4 Data analyses

All data analyses described in this section were done collaboratively with Dr. Paula Satizabal, a geneticist from LEVMA, Universidad de los Andes, Bogota. Only data of the 35 samples from which DNA was extracted were included in the analysis.

8.2.4.1 MtDNA CR sequence analyses

Sequences for the mitochondrial D-loop CR were edited and aligned manually using the software Geneious Pro version 4.8.5 (Drummond et al., 2009). Forward and reverse sequences were confirmed by alignment with each other and a consensus sequence was obtained. Haplotypes were defined using MacClade (Maddison and Maddison, 1992), and for phylogeographic comparisons two consensus regions of 647bp and 401bp were compiled and analysed. The 401bp dataset was used for wider phylogeographic comparisons using previously published CR sequences from India, Indonesia, Thailand and the Philippines (Appendix 9). The consensus regions were compiled, analysed and compared with all sequences available from GenBank, in order to detect shared haplotypes amongst *O. brevirostris* from all regions.

8.2.4.2 Haplotypes

The 647bp database was used in order to understand the microgeographical distribution of different haplotypes along the pools in the Mekong River. A haplotype network was constructed using the statistical parsimony methodology as implemented in the software TCS version 1.21 (Clement et al., 2000). This method estimates an un-rooted tree, providing a 95% plausible set for all sequence type linkages within the tree and considers gaps as a fifth

character state. Haplotype (h) and nucleotide diversities (π) among MRID samples were performed in the program Arlequin version 3.5.1.2 (Excoffier et al., 2005) and restricted to the 647bp sequence dataset.

8.2.4.3 Phylogenetics

Phylogenetic analyses were performed using the substitution model determined by Modeltest version 3.06 (Posada and Crandall, 1998). Phylogenetic reconstructions of Maximum Parsimony, Maximum Likelihood and Neighbour-Joining methods were performed in PAUP v4.0b1 (Swofford, 2002). The rough-toothed dolphin (*Steno bredanensis*) and the Australian snubfin dolphin (*Orcaella heinsohni*) were used as outgroups for these analyses.

8.2.4.4 Microsatellite data

The binning of the microsatellite data to determine the allele number was performed with the software TANDEM (Matschiner and Salzburger, 2009). Arlequin version 3.5.1.2 (Excoffier et al., 2005) was used to calculate the number of alleles (NA), the observed heterozygosity (H_O) and the expected heterozygosity (H_E) for every locus. Microsatellite loci null alleles were tested using Micro-Checker version 2.2.3 (Van Oosterhout et al., 2004).

8.2.4.5 Genetic structure

Previously in Chapter 6, there was a difference found between the prevalence of skin lesions in dolphins from pool 9 compared to pool 1. These pools are geographically isolated. Furthermore, from distribution studies on the MRIDs (Dove, 2008; Ryan et al., 2011), it appears there is restricted movement of dolphins between the following groups of pools: pools 1-3; pools 4-5; pools 6-8; and pool 9. These groups were tested in this study for

evidence of any population structure using Arlequin version 3.5.1.2 (Excoffier et al., 2005) with bootstrap analysis for all possible structural hypotheses. Structure Harvester web version 0.6.92 (Earl and von Holdt, 2012), was used to estimate and summarise structure results using statistic Delta *K*, and final *K* values were obtained after estimating the variance among runs using CLUMPP version 1.1.2 (Jakobsson and Rosenberg, 2007). Structure harvester produces a plot of the mean likelihood values per *K* (number of clusters) as described by Earl and von Holdt (2012). The inference of true *K* was given by the log likelihood for each *K*, lnP(*D*)=ln(*K*). The most likely *K* was chosen using the Evanno Method, looking at Δ*K* by calculating the second order rate of change of lnP(*D*) between the values of *K* (Evanno et al., 2005).

The samples used in this study were summarised according to the GPS coordinates where the carcasses were reported/found (Table 8-3). Where the carcass was downstream from a pool, the closest pool upstream was used as the pool location for this study (Appendix 10). Only one sample was collected from pool 9.

Table 8-3: Proportion of the population sampled per river section

Pools	1 - 3	4 & 5	6, 7 & 8	9	Total
# Dolphins identified (ID)	53	9	20	6	88
Genetic samples	25	3	6	1	35
Percentage of identified dolphins	47	33	30	17	39.8
95%CI	33.3, 61.4	7.5, 70.1	11.9, 54.3	0.4, 64.1	29.5, 50.8

The geographical distances between alleles were compared with the D-*loop* and microsatellite genetic distances using the Mantel Test in Program R (R Development Core Team, 2005).

8.2.4.6 Genetic drift and bottleneck testing

Demographic changes in population size of the MRIDs were estimated using an Extended Bayesian Skyline Plot (EBSP), a multilocus approach implemented in Beast version 1.7.1 (Drummond and Rambaut, 2007; Heled and Drummond, 2008). This coalescent-based method assumes one panmictic population, as reported for the MRIDs by Krützen et al. (2018).

8.2.4.7 Paternity tests

The 35 samples that returned a result in this study were from 35 unique individuals, and the population abundance was 85 dolphins when this study commenced, thus the paternity of the samples were tested using Cervus version 3.0 (Kalinowski et al., 2007; Marshall et al., 1998). The analysis was run with a total population of 120 (85 plus the 35 dolphins sampled), as well as a population of 85. The 35 samples included calves and juveniles (n=28; with 27 calves and 1 juvenile), adult females (n=3), and adult males (n=4). Overall the samples came from 15 males, 19 females, and 1 dolphin calf of unknown sex. The simulations were run with a probability of 0.3 (35/120).

8.2.4.8 Statistical analysis

Levels of polymorphism, Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were estimated using Arlequin, and p-values were adjusted using the Bonferroni

correction. Wright's fixation index F_{ST}, inbreeding coefficient F_{IS}, and analysis of molecular variance (AMOVA) were also estimated in Arlequin. The AMOVA was performed to compare variations within and between groups in order to identify genetic subdivision between the different pools.

To estimate the number of genetic clusters of the MRIDs, and to determine the degree of admixture among the cluster, a Bayesian approach was implemented with the software Structure version 2.3.3 (Pritchard et al., 2000). Ten independent admixture model simulations with 1 million repetitions of Monte Carlo Markov Chain (MCMC) and a burn-in of 100,000 steps were tested per cluster (*K*) evaluated from 1 to 15, using the LOCPRIOR option, in an attempt to measure weak population structure.

The EBSP method was run using 100 million MCMC chain lengths, with a sampling frequency of 1000. Tracer version 1.6 was used for assessing MCMC convergence, ensuring an estimated sample size (ESS) of a minimum of 200 for each parameter (Rambaut and Drummond, 2003; Rambaut et al., 2018). The EBSP calculates the posterior probability of the number of population size changes, where a high posterior probability for zero population size changes corresponds to a constant population size.

In order to understand the likelihood of having infrequent alleles posed by age class, a standard epidemiological contingency table for risk factor analysis was constructed using the methods described previously in Chapter 3, and analysed using a Fisher's exact test with the statistical significance set at $\alpha<0.05$.

8.3 Results

8.3.1 MtDNA CR

A total of 28 (80%, 95%CI 63.1, 91.6) D-*loop* sequences were successfully obtained from the MRID population. A total of 647bp of the CR were analysed. In this study, six new haplotypes (A-F) were identified and defined by seven variable sites (Table 8-4). Three new haplotype sequences for the MRIDs were submitted to GenBank under the accession numbers: MG703248.1, MG703247.1, and MG703246.1 (Caballero et al., 2018a).

The most common haplotype was A (n=11), followed by B (n=9) and C (n=5), with D, E and F each represented once (Table 8-4), with all haplotypes being closely related with only one or a few base-pair differences. For broader phylogeographic comparisons at the species level of *O. brevirostris*, a database set using 401bp was compiled. When assigning haplotype redundancy using the 401bp sequences, haplotypes A, F and E were reduced to haplotypes 1, 27 and 14, respectively. Haplotypes 27 and 14 had not been identified in previous studies. The haplotypes identified in this study were organized according to the geographical location (Table 8-5 and Figure 8-2). One median-joining network cluster was obtained for the MRIDs using the 647bp (Figure 8-3). The maximum-likelihood phylogenetic tree is shown in Figure 8-4, with the MRIDs occupying a unique evolutionary branch compared to the coastal Irrawaddy dolphins.

Table 8-4: Frequency of haplotypes identified by seven variable sites over 647bp of the D-*loop*

Haplotype	**Nucleotide position***							Frequency **(n)**	**Percentage % (95%CI)**
	2	3	3	4	5	5	6		
	0	2	9	5	0	5	0		
	3	4	2	5	1	5	2		
A	T	G	C	T	C	T	G	11	39.3 (21.5, 59.4)
B	.	.	.	.	.	C	.	9	32.1 (15.9, 52.4)
C	.	.	.	C	.	.	.	5	17.9 (6.1, 36.9)
D	.	.	.	.	.	.	A	1	3.6 (0.1, 18.3)
E	.	A	T	C	T	.	.	1	3.6 (0.1, 18.3)
F	C	.	.	C	.	.	.	1	3.6 (0.1, 18.3)

* Each column represents a variable nucleotide position, and the row indicates the nucleotide found at that position in the given haplotype on the left.

Table 8-5: Number of haplotypes identified by pool of origin

Pool	**Haplotypes**						**Total**	
	A	**B**	**C**	**D**	**E**	**F**	**n**	**% (95%CI)**
1	4	2	2		1	1	10	35.7 (18.6, 55.9)
2	2	3					5	17.9 (6.1, 36.9)
3	0	1	1				2	7.1 (0.9, 23.5)
4		1					1	3.6 (0.1, 18.3)
5	1	1					2	7.1 (0.9, 23.5)
6	1	1	2				4	14.3 (4.0, 32.7)
7	2						2	7.1 (0.9, 23.5)
8	1						1	3.6 (0.1, 18.3)
9				1			1	3.6 (0.1, 18.3)
Total	11	9	5	1	1	1	28	100

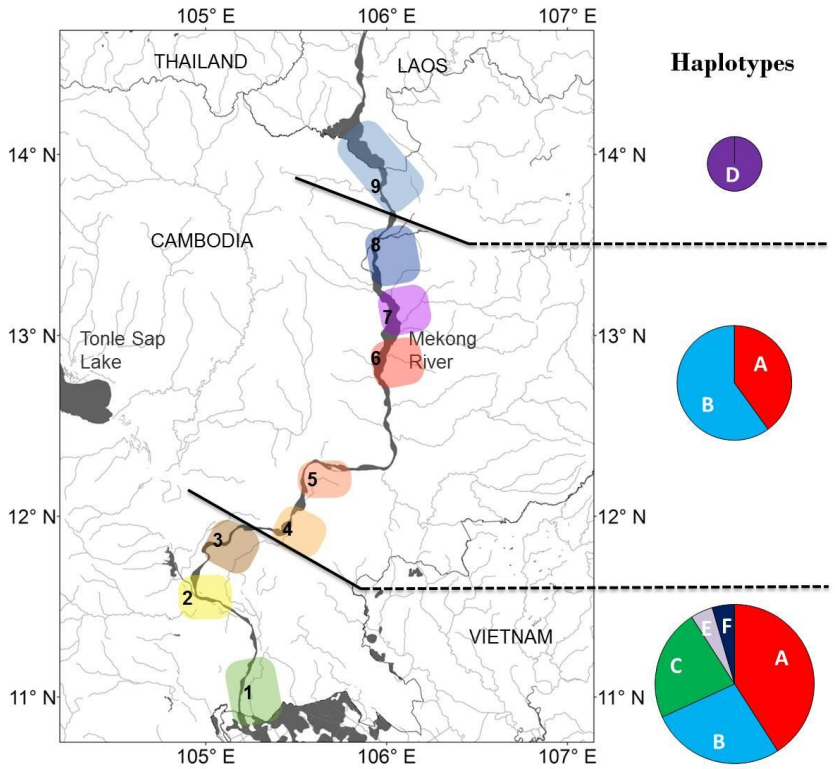


Figure 8-2: Map of the location of the different haplotypes found in the dolphins from the Mekong River.

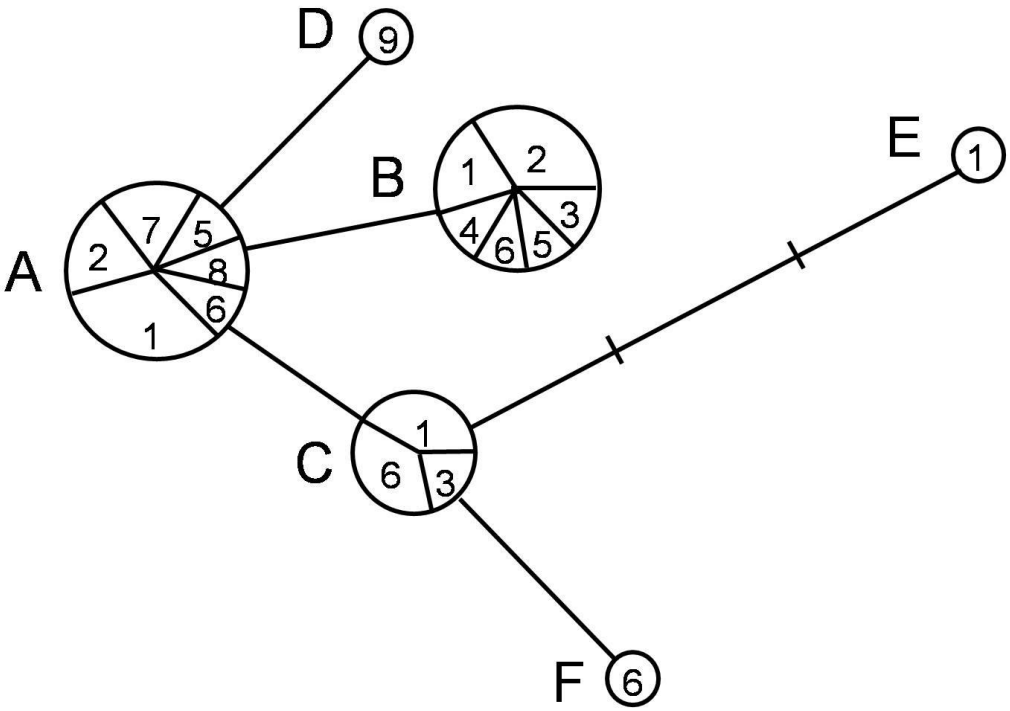


Figure 8-3: Median-joining network for Mekong River Irrawaddy dolphin haplotypes. The size of the circle reflects the frequency of the particular haplotype found in pools (1-9).

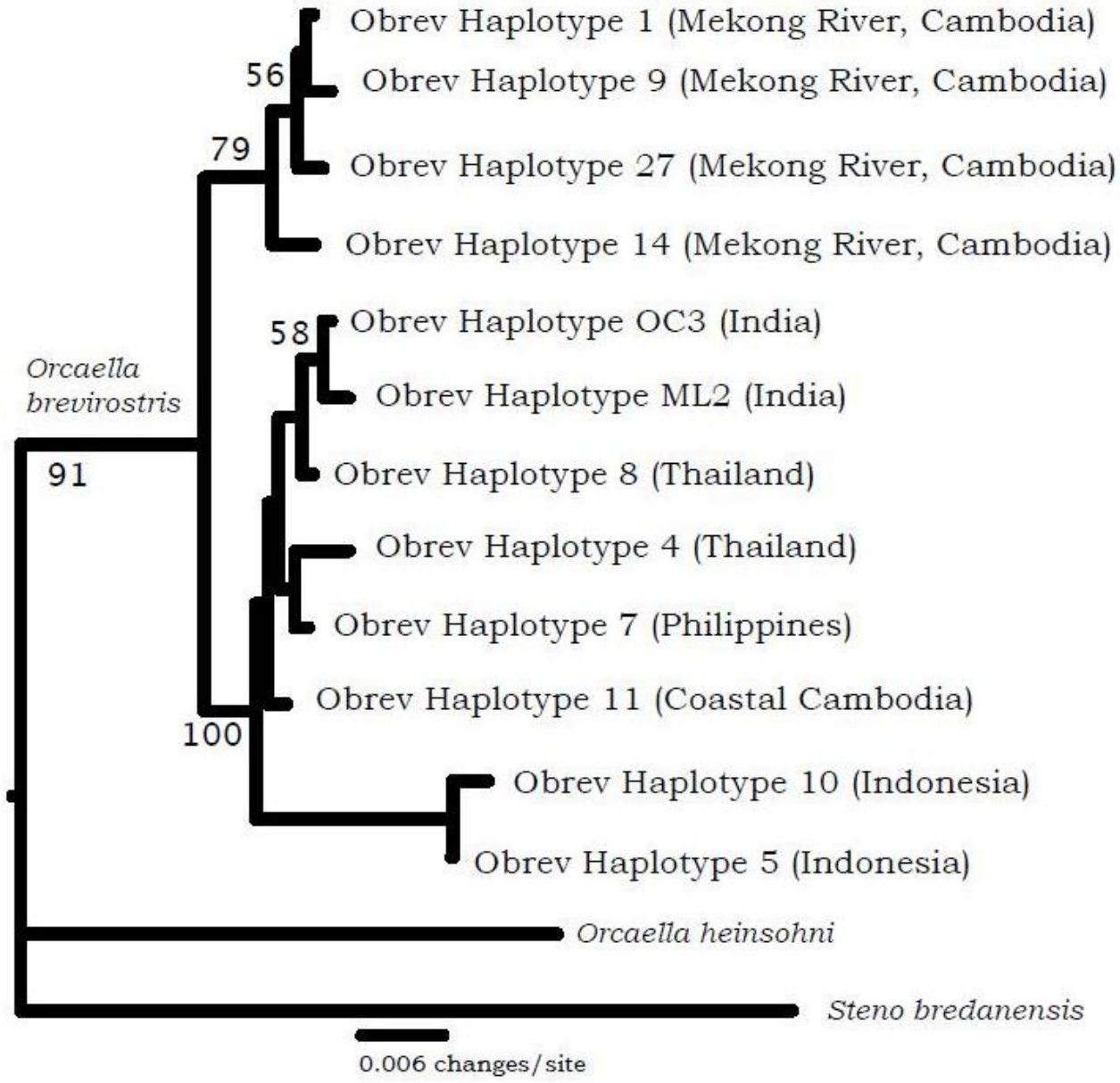


Figure 8-4: Maximum-likelihood phylogenetic reconstruction for *Orcaella brevirostris.*

The average nucleotide diversity (π) was 0.001 (± SD 0.0001) and the average haplotype diversity (h) was 0.812 (± SD 0.333) (Table 8-6).

Table 8-6: D-*loop* number (n) of haplotypes (*h*), nucleotide (π), and haplotype diversity (*Hd*) indices including standard deviation (SD)

Pool	n	*h*	π ± (SD)	*Hd* ± (SD)
1	7	4	0.003 ± 0.002	0.810 ± 0.130
2	5	2	0.001 ± 0.001	0.600 ± 0.175
3	3	3	0.002 ± 0.002	1.000 ± 0.272
4	1	1	0.000 ± 0.000	1.000 ± 0.000
5	2	2	0.002 ± 0.002	1.000 ± 0.500
6	5	4	0.002 ± 0.002	0.900 ± 0.161
7	2	1	0.000 ± 0.000	0.000 ± 0.000
8	1	1	0.000 ± 0.000	1.000 ± 0.000
9	1	1	0.000 ± 0.000	1.000 ± 0.000

8.3.2 Microsatellites

In this study, 32 MRIDs (91.4%, 95%CI 76.9, 98.2) were successfully genotyped for 12 microsatellite loci and 78% (n=25) of the dolphins sampled also had MtDNA D-*loop* sequences obtained. Samples of dolphins from four pools (2, 4, 5 and 8) had lower heterozygosity (HO) than expected heterozygosity (HE). In contrast, the observed heterozygosity was higher than expected in dolphins from pools 1, 3 and 6 (Table 8-7). There was no evidence of deviations from Hardy–Weinberg (H-W) equilibrium or linkage disequilibrium (LD) at $p<0.05$ for any locus.

Table 8-7: Nine microsatellite loci estimates of the number of alleles per locus (NA), mean observed heterozygosity (H_O), and mean expected heterozygosity (H_E) including SD

Pool*	Number of dolphins	NA ± (SD)	H_O ± (SD)	H_E ± (SD)
1	9	7.444 ± 5.364	0.723 ± 0.119	0.610 ± 0.156
2	6	4.875 ± 2.475	0.342 ± 0.114	0.538 ± 0.224
3	3	3.167 ± 1.602	0.694 ± 0.245	0.594 ± 0.122
4	3	2.250 ± 0.500	0.500 ± 0.408	0.533 ± 0.047
5	2	4.833 ± 2.229	0.667 ± 0.516	0.778 ± 0.172
6	5	7.000 ± 4.583	0.656 ± 0.251	0.587 ± 0.192
8	2	4.833 ± 2.229	0.583 ± 0.376	0.722 ± 0.202
9	1	5.800 ± 2.864	1.000 ± 0.000	1.000 ± 0.000

*No microsatellite data were available from samples sourced from dolphins from pool 7.

Nine loci were suitable for use in the analyses, with between two and six alleles per locus (mean/median 4, SD 1.6) (Table 8-8). The remaining three loci were monomorphic. Two samples (Cases 29 and 35), both from pool seven, were excluded from the analysis because of missing data. Loci SGUI011 and SGUI018 showed evidence of null alleles and were also excluded from the analyses (Appendix 11).

In this study, 34% (n=11) of the sampled dead dolphins had Infrequent/Rare (I/R) alleles (Table 8-8). In total, there were 12 I/R alleles found. Of the 11 dolphins with I/R alleles, three were homozygotes, four were heterozygotes with 2 I/R alleles, and four had one I/R allele and one common allele shared with other individuals. Where two or three of the same I/R alleles occurred per locus, the location of origin for these individuals (n=6) was checked. In all of these cases, the individuals were from different pools (1 & 2; 1 & 6; 7 & 9), with only one of the three pairs of animals from pools 1 and 2 likely to have interacted based on geographical distribution.

Table 8-8: Number of alleles found at each locus

Locus	Alleles	Size (bp)
GATA98	3	85, 89, 93*
EV94	6	232, 234, 236, 238, 240, 242
AAT44	4	82, 85, 88*, 97*
PHO142	3	130*, 132, 134
SGUI006	6	212*, 214, 216, 218, 220*, 224
MK6	3	152, 154, 156
SGUI003	6	167*, 169, 171, 173, 175*, 183*
SGUI017	2	166, 168
SGUI016	6	171*, 173*, 175, 177, 181, 189*

*Infrequent/Rare (I/R) alleles (defined as ≤ 3 alleles per loci of that particular size), bp (base pairs)

Although I/R alleles were more common in adults (50%; 95%CI 11.8, 88.2) than in calves (35%; 18.6, 55.9), this difference was not significant (Fishers exact test p=0.652; OR 1.8: 95%CI 0.3, 10.64).

8.3.2.1 Structure

The data in Table 8-9 demonstrates the most likely structure combinations, taking into account the geographical origin of the carcasses sampled. Based on the highest F_{CT} value and the level of significance, there was strongest support for H7 ([1,2,3,4,6,7,8][9] (p=0.03)) from the D-*loop* data. Similarly, the microsatellite data analysis also showed stronger statistical support and highest F_{CT} for H7 ([1,2,3,4,6,7,8][9], (p=0.006)).

Table 8-9: F_{CT} and p-values for the most likely geographical structuring based on geographical pools 1-9

	Most likely hypotheses for grouping pools	F_{CT}	**P-value**
Structure based on the D-*loop*	H1: [1,2,3,4,6,7,8,9]	-	-
	H2: [1,2,3,4][6,7,8][9]	0.396	0.121
	H3: [1][2,3][4][6,7,8][9]	0.222	0.034*
	H4: [1][2][3][4][6][7,8][9]	-	-
	H5: [1,2,3][4][6,7,8][9]	0.222	0.033*
	H6: [1][2,3,4][6,7,8][9]	0.275	0.008*
	H7: [1,2,3,4,6,7,8][9]	0.456	0.030*
	H8: [1,2,3][4,5,6,7,8][9]	0.244	0.06
Structure based on microsatellites	H7: [1,2,3,4,6,7,8][9]	0.243	0.006*
	H8: [1,2,3][4,5,6,7,8][9]	0.036	0.039

*Significant at p <0.05.

The Arlequin AMOVA analysis of microsatellite loci found no significant difference at the p<0.05 level. The overall pairwise fixation index (F_{ST}) was calculated, and there were no significant differences between pools for this population (F_{CT}=0.9; p=0.05), or among populations within pools (F_{SC}=0.5: p=0.33). However, there was a significant difference within the population (F_{ST}=0.02; p=0.003). There was no statistically significant correlation found using the Mantel statistic based on Spearman's rank correlation (rho) for the D-*loop* and geographical location (p=0.428, r=0.004), for geographical distances and microsatellites (p=0.806, r= -0.086), or between the D-*loop* and microsatellites (p=0.749, r= -0.07). The CR AMOVA revealed a lack of population structure among the dead MRIDs found in the various pool locations (p>0.05).

The assignment test analysis showed the highest likelihood at a population structure of K=3 (Appendix 13). Using Bayesian assignment test of the software Structure, lnPr (X|K) for the likely number of populations, lnPr (X|K) decreased with increasing K from K=3. The mean lnPr (X|K) values and their standard deviations are given in Appendix 13. As lnPr (X|K) was highest for K=3, it indicates that some evidence of sub-structuring exists amongst the dolphins sampled. The estimated membership coefficient for all individuals were clustered into a bar plot (Appendix 15), which demonstrated that all individuals in the Mekong River formed one clade comprising three clusters, with any individual within the population having the same probability of being assigned to any of the three clusters.

8.3.3 Genetic drift

Tests for a deficit or excess of heterozygotes within the population were carried out using allele randomisation simulations, and the results were characterized using the FIS statistic (Table 8-10. Negative and non-significant values of F_{IS} were obtained. Significantly positive F_{IS} values indicate a deficit of heterozygotes relative to random mating and negative values indicate an excess of heterozygotes. The results revealed higher heterozygote frequencies than the simulated values for dolphins from all pools on every locus, supporting a heterozygote excess in this population.

Table 8-10: F_{IS} statistic from random allele simulations to test for an excess of heterozygotes in the sampled population

Locus	F_{IS}	P-value
1	-0.20	1
2	-0.22	1
3	-0.56	1
5	0.24	0.139
6	0.30	0.410
7	0.18	0.543
8	0.26	1.0

The EBSP analysis strongly supported a one population size change through time with a posterior probability of 0.921. This was followed by a probability of 0.073 for two population size changes and 0.003 for zero population size changes through time. The supported demographic change in the MRID population size has occurred "recently" (in the past 10,000 years) as depicted in Figure 8-5, with the posterior probability being skewed towards the present, suggesting a recent population decline. In this figure the Y axis represents the population size (number of individuals), and the X axis represents time in years. The black line represents the population size mean derived from the demographic function. The grey shading represents the 95% credible intervals of the population size estimate. The bars correspond to the posterior probability of a population size change through time. Figure 8-5 includes a histogram describing the locations of the demographic functions X-axis points.

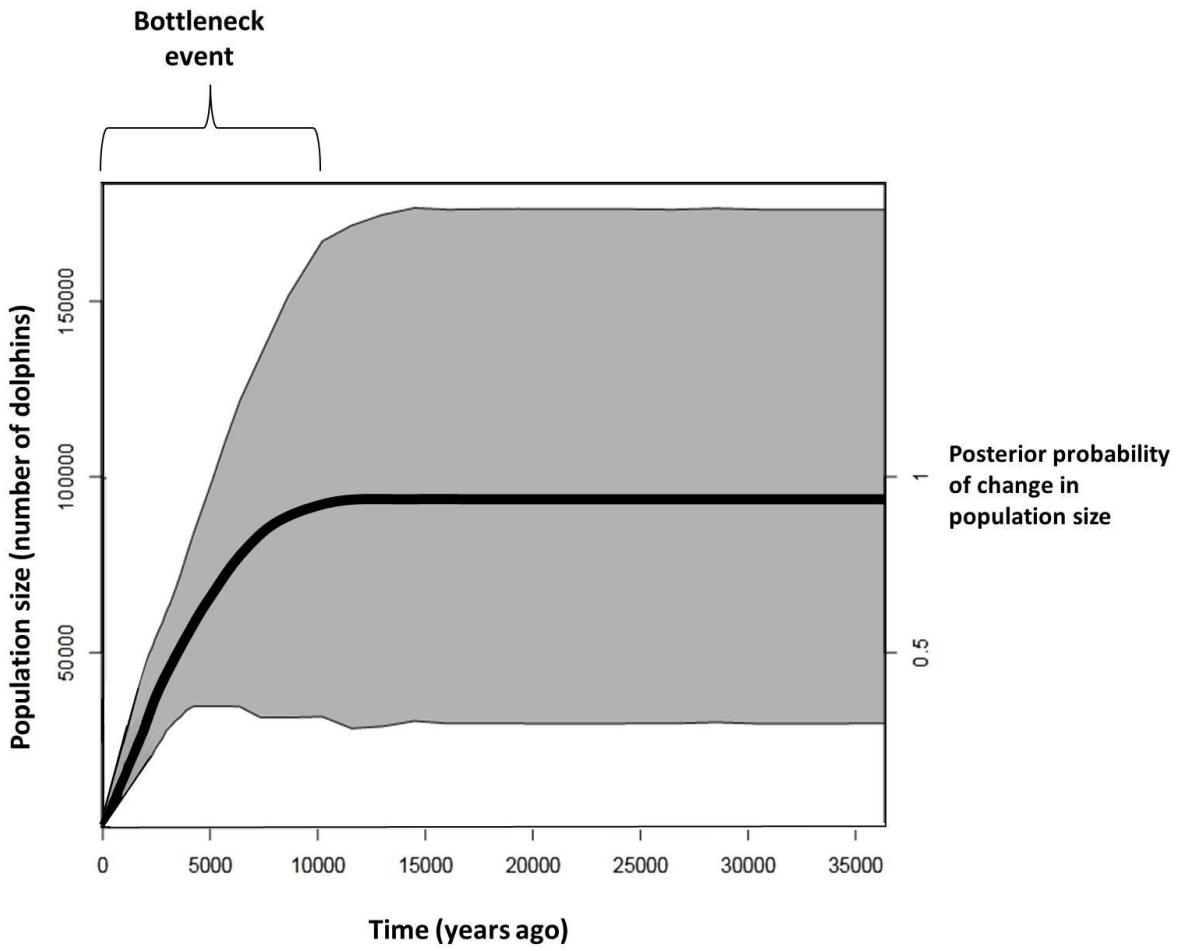


Figure 8-5: Microsatellite multilocus extended Bayesian skyline plot (EBSP).

8.3.4 Paternity test

In the paternity analysis, candidate offspring Case 32 (3 loci) and candidate father Case 27 (3 loci) were both excluded from the analysis because they were typed at fewer than 4 loci. The paternity test results from Cervus 3.0 were inconclusive due to missing genetic data, and thus did not provide any significant results. The analysis suggested Case 13 as the possible paternal match for all samples. However, this result was not significant and may be explained by the lack of data. Additional likely paternities were assigned to three other males (Cases 2, 13 and 27) at the 95% confidence levels.

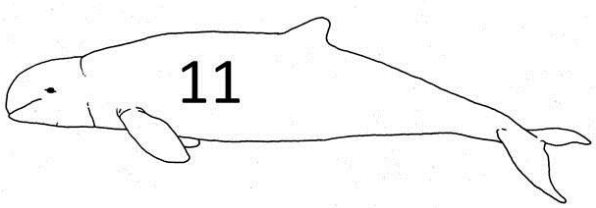
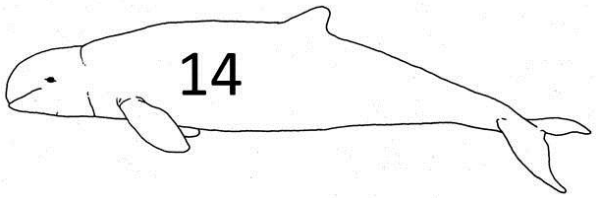
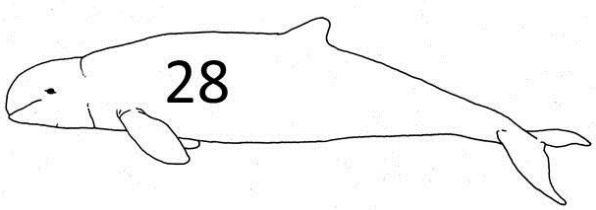
The results for the maternity test revealed that there was a 75% probability that the true assigned mothers were sampled (Table 8-11), and that Case 11 is the possible mother of seven calves (25%), Case 14 of five calves (18%) and Case 28 of one calf (4%) (Table 8-12). To assess the likely matches between mothers and calves, the year the mother died was compared to that of the calves, in addition to the haplotype of the mother and calf, and location data. By doing this three mother - calf pairs were successfully identified (Table 8-11) (Case 11 with Case 09; Case 14 with Case 18; and Case 11 with Case 15). In addition, Case 14 was a possible mother of Case 31, although haplotype data were missing for Case 31.

Table 8-11: Maternity assessment of sampled offspring

Calf case #	Mother case #	Pair confidence	Date calf carcass detected	Date mother carcass detected	H calf	H mother	Pool calf found in	Pool mother found in	Calf morphometrics	
									Length (cm)	**Weight** (kg)
09‡	11	*	31/12/2008	27/05/2009	C	C	1	1	69	3.8
31‡	14	*	5/02/2006	14/06/2007	?	A	4	1	105	13.8
08	11	+	16/12/2005	27/05/2009	A	C	1	1	106	15.2
21	11	*	3/02/2006	27/05/2009	A	C	2	1	107	13.8
23	11	+	1/02/2006	27/05/2009	A	C	2	1	95	10.8
18‡	14	*	3/02/2006	14/06/2007	A	A	1	1	110	15.8
26	11	*	2/03/2008	27/05/2009	B	C	3	1	104	14.5
30	14	+	3/03/2007	14/06/2007	B	A	4	1	100	6
22	14	*	13/02/2006	14/06/2007	B	A	2	1	106	16
24	11	+	13/02/2006	27/05/2009	B	C	2	1	102	11
15‡	11	+	20/05/2008	27/05/2009	C	C	1	1	122	28
19	28	*	13/02/2006	6/05/2007	C	A	1		107	14.6
17	14	+	14/01/2006	14/06/2007	F	A	1	1	77	6.1

‡ Likely match * $p<0.01$, + $p<0.05$, H (haplotype) as indicated in Cervus. Pools are either where carcass was retrieved from, or the closest pool upstream from the carcass

Table 8-12: Mother-calf pairs identified in Cervus

Offspring case #	Candidate mother case #	Pair confidence~
09		*
08		+
21		+
23		+
26		*
24		+
15		+
31		*
18		+
30		+
22		*
17		+
19		+

Number, ~ Pair Confidence, + $p<0.01$,* $p<0.05$ as indicated in Cervus

8.4 Discussion

There were three key findings in this study indicating that: the MRIDs may be an evolutionary significant unit (ESU); genetic drift of the population may be occurring; and there appears to be reproductive failure in the population. These findings are discussed in detail below.

8.4.1 Evolutionarily significant unit

From the results of this study, it appears that the MRIDs belong to an evolutionarily significant unit (ESU) (Moritz, 1994; Ryder, 1986). This is because mtDNA CR sequences from the MRIDs were found to be grouped into a distinct reciprocal monophyletic clade, separated from other freshwater and coastal populations of Irrawaddy dolphins with high bootstrap support. This was similar to the findings reported by Krützen et al. (2018). In order to attempt to confirm the ESU status of the MRID population, a collaborative study was initiated in which the divergence of allele frequencies from this study and other populations of *O. brevirostris* were examined. This collaborative study resulted in the publication of the manuscript by Caballero et al. (2018a). In that study we found the results did not support reciprocal monophyly, as initially suggested by the work in this chapter. This is likely because smaller fragments (400bp and 486bp) were used in the Caballero et al. (2018a) study compared to the 647bp used in the current study. Thus, based on this, and the results in this chapter, it is likely that the MRIDS do belong to a unique ESU. ESUs distinguish a geographically discrete set of historically isolated populations, potentially defined from natural history information, morphometrics, range and distribution data, and genetic data (Ryder 1986). The pattern of reciprocal monophyly of mtDNA haplotypes meets the ESU definition based on one of the criteria described by Moritz (1994). However, further research utilising the divergence of allele frequencies at nuclear loci (microsatellites) from other *O. brevirostris* populations is required for confirmation of the ESU status for the MRIDs.

In Caballero et al. (2018a) one haplotype in the MRIDs was found to be shared with dolphins from the Gulf of Thailand, which may suggest, from an evolutionary perspective, that

freshwater colonisation by coastal Irrawaddy dolphins may have occurred in relatively recent geological times. A molecular clock was not used in this study, however based on the study by Caballero et al. (2017) where a molecular clock was incorporated, divergence may have occured between 100,000-300,000 years ago. This was in contrast to that found by Krützen et al. (2018), who suggested a long-standing isolation of the MRIDs from other coastal Irrawaddy dolphin populations. The difference between these findings may be due to the fact that in Caballero et al. 2018a, we were able to use a larger fragment of the mitochondrial control region control region (486bp) compared to that (384bp) used by Krützen et al. (2018). This longer CR fragment permitted further differentiation of haplotypes compared to that undertaken by Krützen et al. (2018). In Caballero et al. (2018a) we also found significant levels of genetic differentiation between coastal and riverine populations and between sampling locations, suggesting that, from a conservation management perspective, the MRIDs should be managed as a separate entity (Moritz, 1994).

8.4.1.1 Genetic diversity

In this study, six new haplotypes were defined, suggesting that haplotype diversity appears high for the MRIDs. However, when compared to the previous work by Beasley et al. (2005) using a shorter CR fragment, the six haplotypes were reduced to three haplotypes, two of which were newly defined in this study. One haplotype (Hap 9), previously reported by Beasley et al. (2005), was not found in the current study. The haplotype diversity in the MRIDs was found to be half of that found in the population in the Gulf of Thailand, but greater than that found in Chilika Lake (Caballero et al., 2018a). Previously only two CR haplotypes were reported for the MRID (Beasley et al., 2005), comparable to that found by Jayasankar et al. (2011) in the Chilika Lake Irrawaddy dolphins. The samples used in the

current study and in that described by Caballero et al. (2018a), represented 40 and 32%, respectively of the estimated total population of MRIDs in 2010, which suggests reduced genetic diversity, both at the mitochondial and nuclear levels. This was similar to the findings reported by Krützen et al. (2018).

Based on the number of haplotypes found in the MRIDs, genetic diversity at the mitochondrial level appears to be relatively high for this population, and similar to that found in other coastal and riverine dolphins, such as *S. guianensis* and *S. fluviatilis* (Caballero et al., 2007). Reduced genetic diversity in the MRID population with a small population abundance would usually be expected, however, there is a time lag between changes in abundance and reductions in genetic diversity (Caballero et al., 2018a). This is due to processes such as genetic drift, which changes genetic diversity and gene frequencies slower than that which would occur with rapid changes in abundance (Caballero et al., 2018a). Additionally, Schou et al. (2017) found that in small populations, genetic diversity may remain high as the rate of loss occurs much slower than predicted. Furthermore, the apparent high mtDNA genetic diversity found in this study may be the result of historical genetic diversity remaining in the population, and might not be reflective of the current genetic diversity status (Schou et al., 2017).

8.4.2 Genetic drift

In this study, there were three findings that give support to the hypothesis of genetic drift occurring in the MRIDS.

8.4.2.1 Recent population size reduction

Firstly, the skyline plot (Figure 8-5) demonstrated a recent population size reduction. Populations naturally experience fluctuations in size and range over time (Alasaad et al., 2011), and if the fluctuations are pronounced enough, populations may become very small, experiencing a bottleneck, or they may even become extinct (Morris and Doak, 2002; Peery et al., 2012). If the population size remains small for several generations, significant loss of genetic variation will result (Amos, 1996). In this study, there was insufficient data to conclude if a bottleneck had occurred in the MRID. This was similar to that reported by Krützen et al. (2018). This is not surprising as Peery et al. (2012) reported in their review on bottleneck studies that microsatellite-based bottleneck tests often do not detect bottlenecks in vertebrate populations known to have experienced declines in very recent times, as was shown to be the case for the MRIDs in this study. Due to the association with bottleneck events and increased extinction risk, the identification of a population bottleneck is generally considered critical for conservation of populations that have experienced significant reductions in abundance (Peery et al., 2012) and, as such, further research is required, particularly given the likelihood of genetic drift occurring in the MRIDs. Additionally, the lack of recruitment in the MRID population may further delay demonstration of a bottleneck in this population, and so to avoid a Type I error, these results should be interpreted with caution, and the possible occurrence of a bottleneck in the MRIDs remains likely, yet uncertain.

8.4.2.2 Population structure change

Secondly, there was a high likelihood of a chronological change in the population structure occurring currently, diverging from H7 to H8. Figure 8-6 is a graphical chronological

depiction of the structure results, demonstrating that H8 ([1,2,3][4,5,6,7,8][9]) is more likely to be a newer divergence of population structure, which, although not statistically significant (p=0.06), is biologically important. During the photo-identification study, possible geographic isolation in certain riverine pools (Chapter 6) were noted for the MRIDs, with limited movement from pools 1-3, 4-8, and 9 (Ryan et al., 2011). This observation fits with the results obtained in the study reported in this chapter.

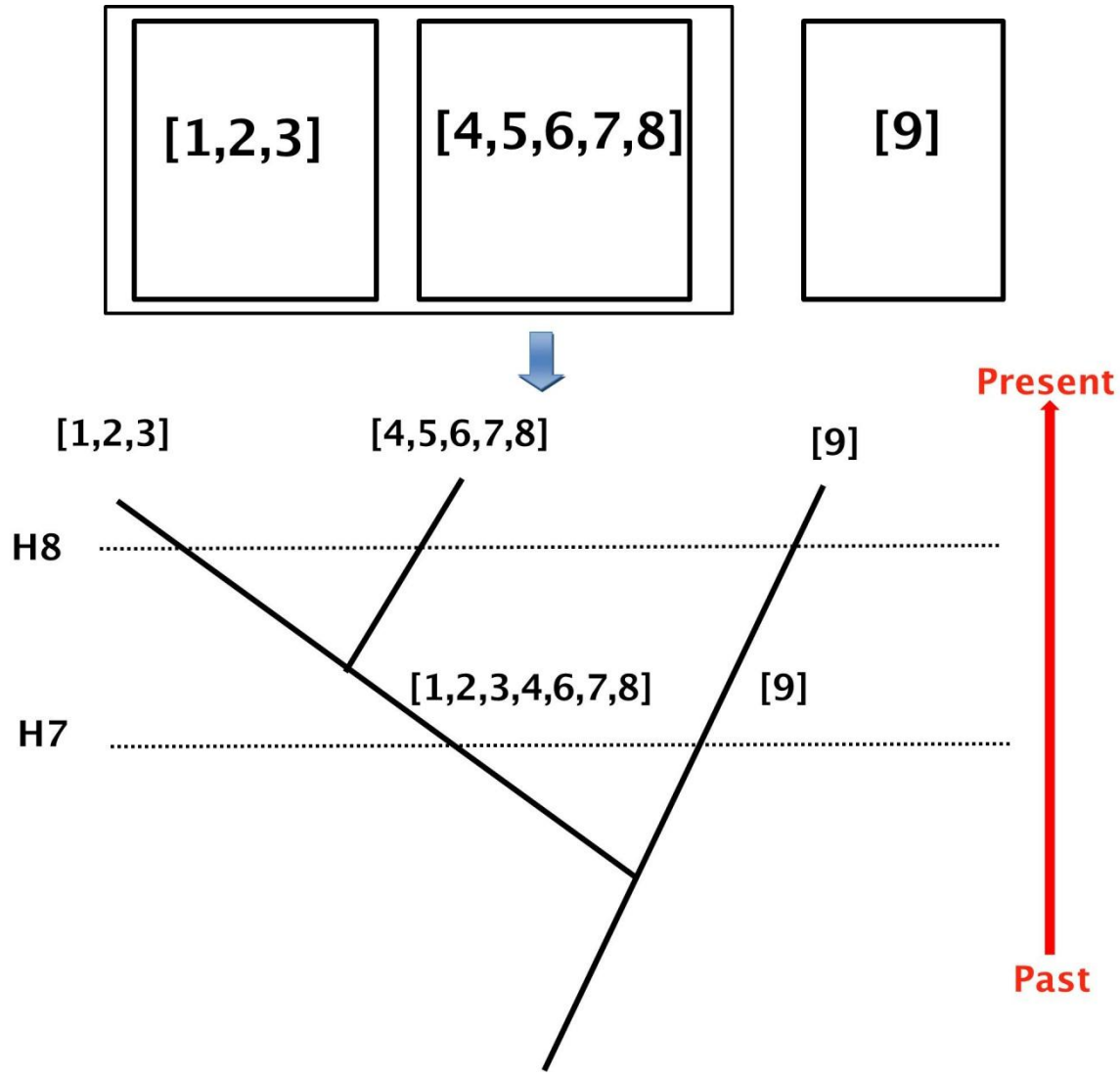


Figure 8-6: Chronological depiction of the most likely population structure.

In contrast to the results presented in Figure 8-6, analysis of the population structure for the CR and the microsatellite loci supported population panmixia for the MRIDs, suggesting that all dolphins in all pools belong to one population unit. This was also reported by Krützen et al. (2018). A possible explanation for this discrepancy is that geographical isolation is very recent, such that it cannot yet be detected by these molecular markers. Thus, it is most likely these dolphins are from a single founding population. Due to contrasting results obtained in this study, further research into the population structuring is required to confirm if the hypothesis of recent divergence is substantiated.

8.4.2.3 Infrequent/Rare alleles

Finally, there was an excess of I/R alleles found, particularly in the adults compared to the calves. This suggests that there may be genetic drift occurring in this population. This may be as a result of the indiscriminate slaughter of the MRID population during the war in the 1970s for oil and for target practice (Beasley, 2007; Beasley et al., 2013), which drastically reduced the population from around a thousand individuals (Krützen et al. 2018; Limsong et al., 2017), to around 200 in the 1990s (Baird and Beasley, 2005). This "chance" event may have changed the allele frequency in the MRID population that may not be reflective of the original population prior to the war. From this study it appears that some of these surviving dolphins had I/R alleles that have remained in the population. Evidence of this was found in this study, with 41% of the sampled dolphins having I/R alleles, with a high proportion of I/R alleles present across all loci. These I/R alleles were defined as unique alleles that occurred at a very low frequency within a particular locus. As I had access to samples from two generations (adults and calves), I was able to observe the frequency of the I/R alleles in the second generation, and confirm that the I/R alleles were being lost in this generation. The

probability of adults having I/R alleles was 1.8 times greater than that for calves, with 50% of adults having I/R alleles, compared to 36% of calves. Hoffmann and Willi (2008) described such serial sampling across a known number of generations to trace the changes in the frequency of I/R alleles, which can also give higher precision to tracking population declines. They considered this method to be more sensitive than classic moment based methods, because genetic drift reduces the number of I/R alleles more strongly than it affects heterozygosity that is dominated by alleles of intermediate frequency (Hoffmann and Willi, 2008). Crosses between individuals with I/R alleles and those without can result in a loss in fitness due to either the disruption of intrinsic interactions between genes, or the disruption of extrinsic interactions between genes and the environment (Edmands, 2007). Based on the results in this study there is clear evidence that genetic drift may be occurring in the MRID population with the frequency of I/R alleles being lower in the second generation.

The change in the I/R allele frequency between adults and calves demonstrates that there appears to be allele drop-out occurring in subsequent generations. With genetic drift, either situation: allele drop-out or allele fixation, would be expected to occur. Usually in a healthy population calves would be expected to survive and subsequently add to the genetic pool through random mating (Figure 8-7) and recruitment (Figure 8-8). However, with the MRIDs there is virtually no recruitment into the population, thus there is no further genetic assortment occurring, creating a 'genetic time capsule' (Figure 8-9). A 'genetic time capsule' was defined in this study as a population frozen in time genetically. With the MRIDs there is no recruitment into the population, thus there is no new genetic material being added to the population, creating a unique anomaly event referred to by the author as a 'genetic time capsule' in this population.

Alleles unique to a population are more likely to be lost by the effects of random genetic drift, which can exacerbate the rate of loss of genetic diversity in a small population (Hartl and Clark, 2007). However, as a result of the ‘genetic time capsule’ within the MRID population there is no allelic drop-out of the I/R alleles due to genetic drift, thus the I/R alleles give an impression of higher genetic diversity in this population, resulting in overestimation of the genetic diversity. Thus, a holistic approach is required when looking at the genetics of the MRIDs.

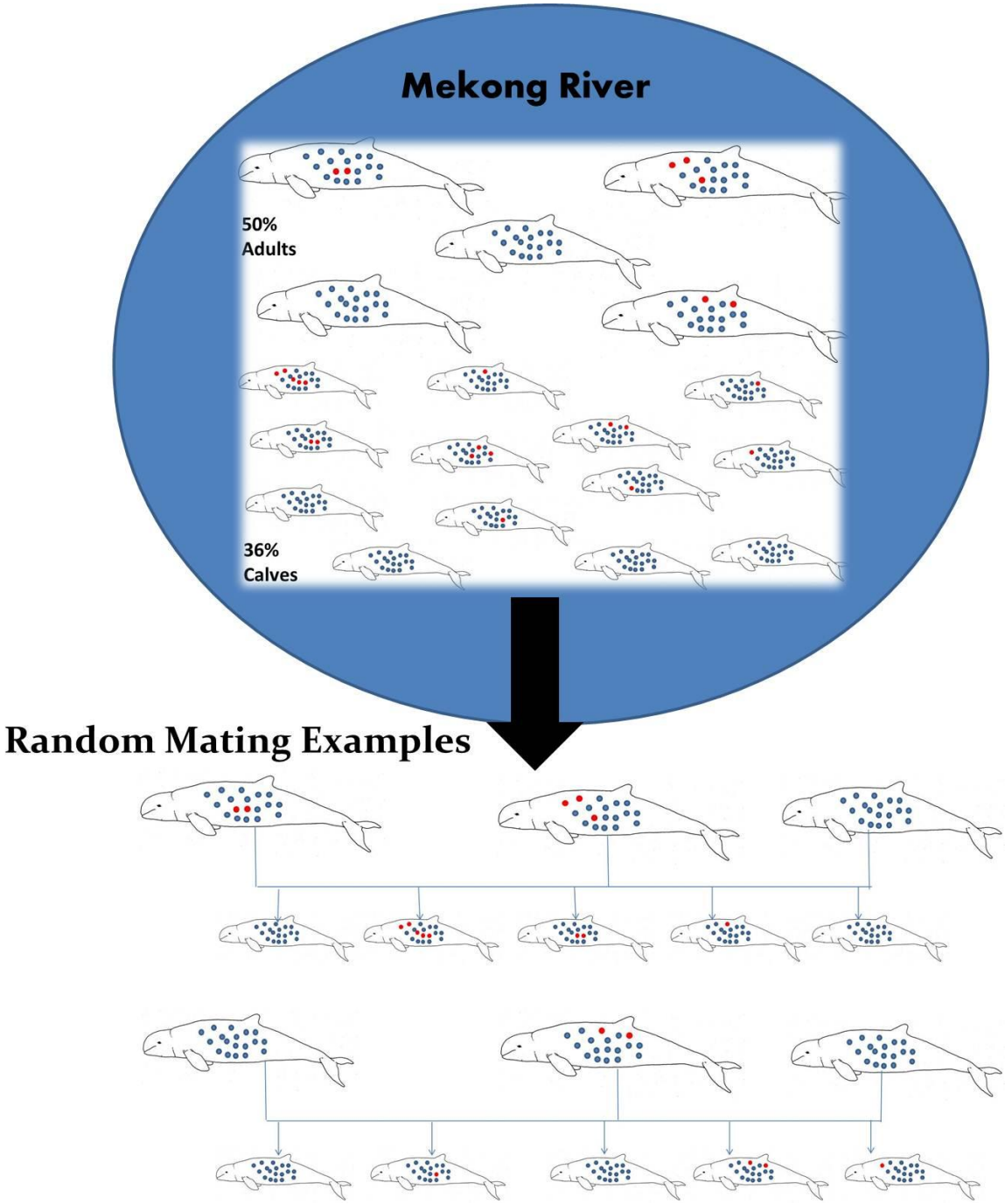


Figure 8-7: Schematic of Infrequent/Rare alleles (red dots) in the Mekong River Irrawaddy dolphin population and examples of random mating events showing I/R allele assortment in calves.

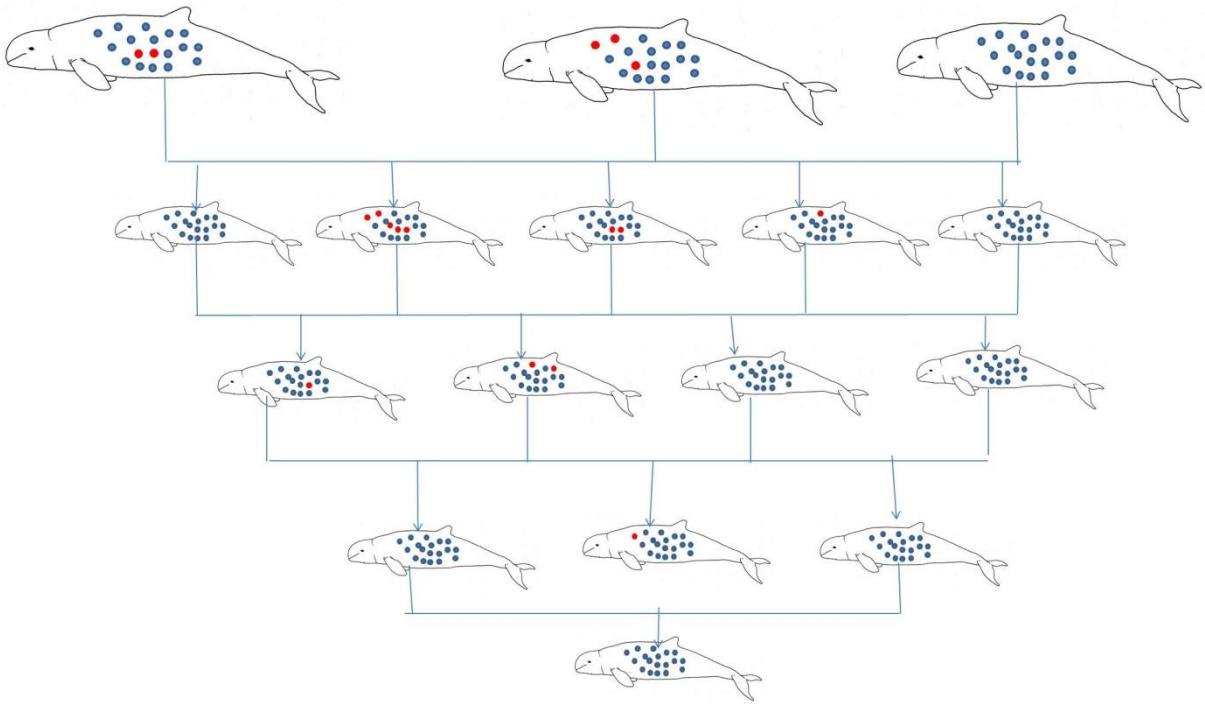


Figure 8-8: Infrequent/Rare alleles in a healthy population with recruitment.

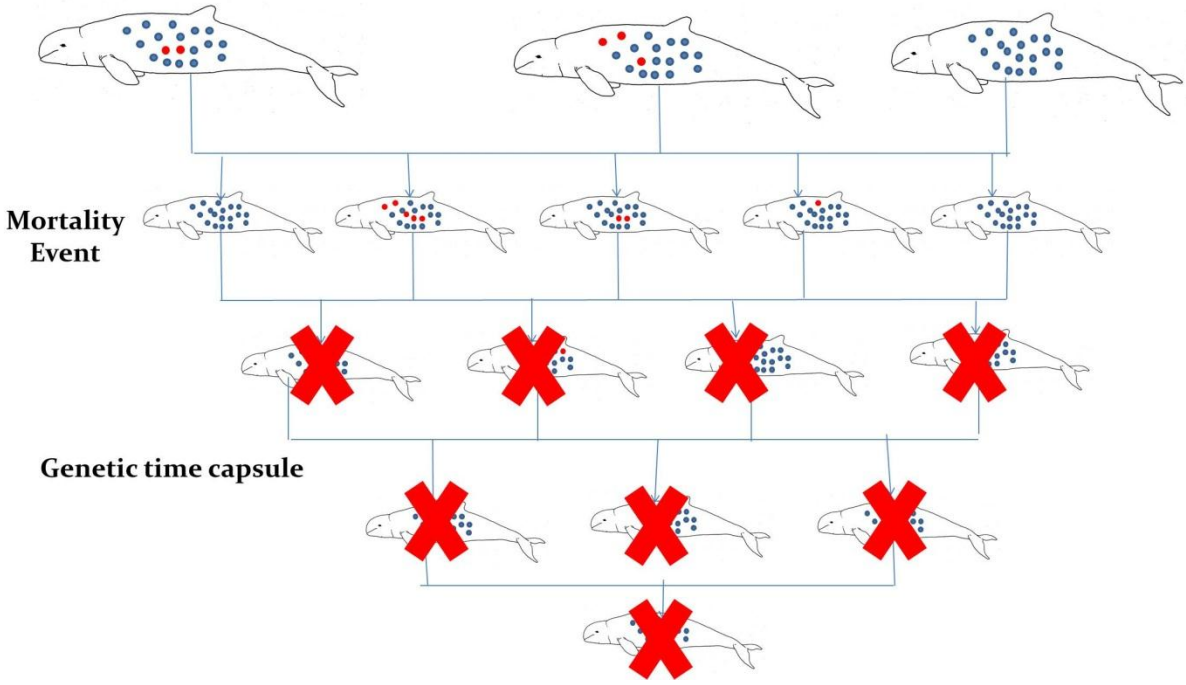


Figure 8-9: Infrequent/Rare alleles in the Mekong River Irrawaddy dolphin population with no recruitment creating a ‘genetic time capsule’.

8.4.3 Reproduction failure

The results in this study demonstrated that only a few dolphins were breeding in the population. Only a few males were siring calves and the calves born were from a small number of mothers. Assuming an equal proportion of males to females, half of the adults in the population are likely to be female (n~42). As mortality of neonatal calves has been documented since 2001, with minimal recruitment, it was assumed that the majority of these females were of breeding age (>6 years, based on data from *O. heinsohni,* (Marsh et al., 1989). The results in this study indicate that only a few females are actually breeding and that breeding females are genetically related. As the population is age skewed (Ryan et al., 2011), it may be possible that older females are grandmothers, and no longer breeding. This grandmother hypothesis is discussed further.

8.4.3.1 Grandmother hypothesis

Reproductive senescence or "menopause" occurs in several cetacean species. Female short-finned pilot whales (*Globicephala macrorhynchus*), killer whales (*O. orca*) and sperm whales (*Physeter macrocephalus*) have menopause with similar attributes to human females (Karniski, 2019; Kasuya and Marsh, 1984; Marsh and Kasuya, 1986; Morell, 2018; Olesiuk et al., 1990). In all these species, reproduction ceases at approximately 40 years of age, although females routinely live on for several more decades (McAuliffe and Whitehead, 2005; Morell, 2018). This "grandmother" hypothesis warrants further investigation in the MRID population, as it is unknown whether or not the female dolphins die at the same age as their male counterparts, or go into reproductive senescence/menopause. However, at least two presumably aged adult females that died, were gravid at the time of death, one reported in Chapter 4 and one in a study by Beasley et al. (2009), suggesting that some may remain

capable of becoming pregnant up until their death. These females had very worn teeth, indicative of older animals as reported in Atlantic spotted dolphin (*Stenella frontalis)* and bottlenose dolphins (*T. truncatus*) *(*Loch and Simões-Lopes, 2012). However, this is not conclusive as old long-beaked common dolphins (*Delphinus capensis*), Fraser's dolphin (*Lagenodelphis hosei*) and Guiana dolphin (*S. guianensis*) have no dental attrition with older age (Loch and Simões-Lopes, 2012). Thus, further research on aging of MRIDs is required, in order to assess how old adult females are when they die, compared to the males in the population.

The findings in this study are biologically significant on at least two major aspects. Firstly, it demonstrates that a large proportion of the sampled females are not breeding successfully, and secondly, that the population may be in a more critical state than previously believed. Smith (2017) maintained that while females are still reproducing there is hope for recovery of the MRID population, providing the aetiology of calf mortality is identified and corrected before the population is too old. However, if a high proportion of females are in reproductive senescence, the population may already be too aged to survive. Furthermore, the survival likelihood of calves born to aged mothers is reduced (Karniski, 2019; Morell, 2018). The reason for this is not clear, although it may be related to a greater chance of genetic mutations and chromosomal abnormalities in older mothers (Karniski et al., 2018; Karniski, 2019), as occurs in humans (Chen et al., 2017a; Kim et al., 2013) and other species such as bovines (Takeo et al., 2013). This would further add to the limited recruitment success in this population.

8.4.3.2 Implications of recruitment failure for the MRIDs

The recruitment failure in the MRID population, defined here as a lack of calves surviving until sexual maturity, has several important and noteworthy genetic implications identified by this study that need to be carefully considered. Recruitment failure may:

1. Result in the I/R alleles appearing to be maintained in the population and adding to the genetic diversity.
2. Mask the occurrence of inbreeding within this small population, as for inbreeding to be apparent you need a successful breeding population adding to the gene pool.
3. Result in no new genotypes being added to the gene pool, creating a 'genetic time capsule'.

8.4.4 Incidental findings

8.4.4.1 Inbreeding/outbreeding

In this study, it was expected that the population would have a low genetic diversity and be suffering from inbreeding due to the small population size. However, the effects of inbreeding/inbreeding depression are unlikely to be seen in this population until the progeny survive and recruitment back into the population occurs with successful breeding. In contrast, the excess of heterozygosity found in this study may suggest that outbreeding depression is occurring in the population as suggested by Coltman et al. (1998) and Finke and Jetschke (1999). Outbreeding depression is defined as a reduction in reproductive fitness (reduced ability to mate, fertilise, produce offspring, survive, or reproduce) of distantly related individuals caused by the disruption of locally or intrinsically co-adapted gene complexes, that occurs in the first or later generations following crossing of these different individuals (Frankham et al., 2011; Templeton, 1986; Templeton et al., 1986). Outbreeding depression therefore generally refers to the reduced fitness of progeny of distantly related individuals

within a species (Marshall and Spalton, 2000). The consequences of outbreeding on the MRIDs could be far more severe than inbreeding. For example, Houde (2010) found that outbreeding for one or two generations resulted in outbreeding depression. Finke and Jetschke (1999) also found that outbreeding resulted in a decrease in survivability. Edmands (2007) explained how this occurs, with the first generation outbred progeny usually not affected by outbreeding depression because both parental sets of co-adapted gene complexes are inherited intact. However, the recombination of genetic information in the generation of these progeny could break down these co-adapted gene complexes (Houde, 2010). As a consequence outbreeding depression and therefore fitness reductions are delayed until the F_2 subsequent progeny generations, when deleterious interactions between homozygous loci become exposed (Edmands, 2007; Houde, 2010). This was demonstrated in a study by Goldberg et al. (2005) who found that the disruption of co-adapted gene complexes occurred as a result of meiosis and recombination between chromosomes inherited from parents of different lineages in the F_1 generation. Furthermore, inbreeding and/or outbreeding typically interact with demography by reducing fecundity, juvenile survival and lifespan (Edmands, 2007; Finke and Jetschke, 1999; Frankham and Ralls, 1998; Marshall and Spalton, 2000).

In this study, the inbreeding coefficient (F_{IS}) was negative. Finke and Jetschke (1999) suggested that disassortative mating can result in outbreeding, with a negative inbreeding coefficient (Finke and Jetschke, 1999). Disassortative mating may have occurred in the MRIDs following the indiscriminate killing of most of the population in the late 1970s (Beasley, 2007). This was demonstrated in a study by Allendorf and Hard (2009) where the harvest of wild animals resulted in human-induced evolution caused by unnatural selection.

8.4.4.2 Implications of this study for conservation

Genetic factors must be considered when devising recovery plans for threatened/endangered species, such as the MRIDs (Frankham and Ralls, 1998). Population genetic tools, such as those applied in this study, should be used in conjunction with health assessments (Chapters 3-7) to determine effective conservation strategies and to optimise conservation efforts for threatened populations (Hohenlohe et al., 2021; Frankham et al., 2019; Leigh et al., 2012). This is critical in the MRIDs, as they have suffered a dramatic population decline as demonstrated both in this study and that of Krützen et al. (2018). Integrating genetic considerations ensures that ecological triage can be maximised to guarantee the best possible survival probabilities for the MRIDs, whilst prioritising the preservation of genetic diversity.

8.4.5 Limitations

Notwithstanding these findings, there were four main limitations in this study.

Firstly, only samples from dead dolphins were used in the analysis which could bias the genetic results, particularly if particular genotypes are associated with increased risk of mortality. Further research should focus on biopsy samples collected from live dolphins, as described in a study by Charlton-Robb et al. (2011) on Burrunan dolphins (*Tursiops australis* sp.), to extend the sample set and reduce this bias, and to better ascertain the paternity picture investigated in this study.

Secondly, the degradation of samples and DNA, particularly in older samples that had been stored for many years prior to analysis, may have hindered the ability to obtain adequate data from each sample analysed, resulting in the exclusion of some dolphins from the final analysis.

Thirdly, this study was limited to only one dolphin from pool 9, which could also bias the data, particularly with the structure, as this dolphin had a unique haplotype. As such the structure data should be treated with caution, and future analyses should prioritise collection of samples from any remaining dolphins resident in pool 9, and from historical samples of dolphins that were from pool 9.

Finally, only nine loci were used in this study, and hence estimates of inbreeding and outbreeding provided by heterozygosity are relatively crude, yet the results in this study still indicate that outbreeding depression should be considered a real threat to the survival of this population, although further research is required to substantiate this.

8.5 Conclusions

The MRID population is at risk of losing its remaining genetic diversity over time due to genetic drift. This, together with a high mortality rate in the MRID population for at least the past 2 decades, and no recruitment into the population, may help explain the high level of genetic variability found. The 'genetic time capsule' in the MRIDs demonstrates that there is limited opportunity for the genetic variation to decrease, as would be expected in a population of only 85 individuals. However, population abundance in conservation genetics is not representative of the number of dolphins that pass on their genes to the next generation, defined as the effective population size (N_e). In the MRIDs as the recruitment rate is 0.001 (Ryan et al., 2011), N_e is currently <1, thus the MRIDS may have, unfortunately, already become functionally extinct.

This chapter completes the investigative studies on the MRIDs. The following discussion chapter links in all my findings from Chapters 3-8 into a causal web of association. The main findings from all the studies in this thesis are highlighted, and their limitations defined, in order to understand the factors associated with mortality in the MRIDs. This is then used to determine if it is possible to reduce this mortality, and reverse the decline and inevitable extirpation of this unique population.

CHAPTER NINE

General discussion

9.1 Introduction

The results of the studies presented in this thesis highlight the concern for the long-term survival of the MRID population. The inevitable extirpation of this unique population is shared by others in the literature (Beasley et al., 2013; Krützen et al., 2018, Caballero et al., 2018a); however this population may already be 'functionally extinct', as discussed in the previous chapter.

A complex history of the MRIDs being used as target practice in the war, as well as hunted for oil in lamps (Beasley, 2007; Krützen et al., 2018), together with exposure to anthropogenic stressors, including interaction with fishing activities (Chapter 3), and environmental contaminants (Chapter 7), coupled with immunosuppression and disease emergence (Chapters 4-6), has resulted in favourable conditions for increasing the risk of mortality in this population. These complex interactions are summarised in the figures in Appendices 15 and 16. The results presented in this thesis demonstrate that multiple stressors, most-likely acting synergistically, are contributing to the mortality and population decline of the MRIDs (Appendix 16). Woinarski et al. (2017) found similar results in three endemic Australian vertebrate species: a bat – the Christmas Island pipistrelle (*Pipistrellus murrayi*); a rodent - the Bramble Cay melomys (*Melomys rubicola*); and a reptile - the Christmas Island forest skink (*Emoia nativitatis*). These three species became extinct between 2009 and 2014, with all three extinctions being predictable and probably preventable, if intervention had been undertaken early in the population decline. If interventions are undertaken with the MRIDs,

and these interventions are successful, then potentially it may not be too late to prevent the extirpation of this unique population.

9.1.1 Hypothesis of this study

This study was designed to assess the hypothesis that disease, together with interaction with fishery activities, and genetic factors were contributing to mortality and driving the MRID population decline and potentially the extirpation of the species from the Mekong River. The null hypothesis was rejected in this study based on several key findings. These are summarised below:

1. There was evidence of a dual causality associated with mortality in the MRIDs (Chapter 3). These findings were significant in the different age classes: neck lesions were associated with dead calves and gillnet entanglements were associated with dead adult dolphins.
2. Deceased MRIDs were found to have infections and disease pathology associated with opportunistic pathogens that were most likely associated with their mortality (Chapter 4). Pathological evidence of disease was confirmed in 71% of the necropsied cases, with two cases revealing a strong likelihood of vertical transmission of infection *in utero.*
3. A significant proportion of dead calves were found to have neck lesions characteristic of necrotising fasciitis, and there was a high likelihood of this disease being attributable to their mortality (Chapter 5).
4. A significant proportion of the MRID population (42%) were found to have been affected by skin lesions over a three year period (Chapter 6), suggestive of underlying health concerns, endemic disease and immunosuppression in the population.

5. Deceased calves (predominantly neonates) had levels of POPs equivalent to that found in the adult males in this population (Chapter 7). Several contaminants were found in sufficient concentrations to cause adverse health effects on the population, potentially resulting in immunosuppression, aggression and pathological skin lesions.
6. Infrequent/Rare alleles were found in 34% of the MRIDs sampled (Chapter 8), providing evidence for genetic drift occurring in the population. The population had evidence of outbreeding depression, a phenomenon not previously reported in dolphins.

9.2 Key findings of this study

In this study, the key findings significantly associated with mortality in the MRID population was disease in calves, followed by gillnet entanglement in adult dolphins. These are discussed in more detail below.

9.2.1 Neck lesions in calves

This study provides the first comprehensive review of the neck lesions found in dead MRID calves. I considered these lesions were most likely necrotising fasciitis, with the underlying pathogenesis associated with immunosuppression, infection with opportunistic pathogens and trauma. Necrotising fasciitis was diagnosed based on: the gross pathology observed; the multitude of potentially causative organisms cultured; the high proportion of dead calves with this condition; and the lack of consistent histological changes. These results were in accordance with those of previous studies of necrotising fasciitis in other species (Allender et al., 2009; Bain, 1999; Bishop et al., 2007; Cui et al., 2007; Dale et al., 1999; Gangopadhyay et al., 2008; Maynor and Kulkarni, 2011; Plavec et al., 2016; Schro et al., 2019; Shindo et al., 1997; Wong, 2003).

The health status of the MRIDs was shown to be affected in the studies presented in this thesis, as well as in other studies (Caballero et al., 2018a; Krützen et al., 2018; Schnitzler et al., 2021). However, dolphin population declines associated with disease have not been commonly described, despite some dolphin populations reportedly being driven to extinction (Choudhury et al., 2019; Li, 2020). Although several studies have documented the presence of different diseases in dolphin stranding events (Pautasso et al., 2019; Vargas-Castro et al., 2020), the current study represents one of the first studies documenting the decline of a dolphin population largely due to disease.

9.2.2 Fisheries induced mortality in adults

Since 2006 measures to reduce mortality in the MRIDs have focused on restrictions of gillnets (Beasley, 2007; Limsong et al., 2017), with enhanced conservation efforts occurring from 2009 through enforcing restriction on the use of gillnets (CSG, 2012a; Gulland, 2009; Reeves et al., 2009; Smith, 2017; Limsong et al., 2017; Thomas and Gulland, 2017). The measures applied from 2006-2008 resulted in a significant reduction in adult mortalities (Chapter 3); however, in contrast, there was no concurrent significant reduction in calf mortality. These results also validated the research findings of Dove et al. (2007) and Beasley (2007), although were in contrast to those reported by Reeves et al. (2009), Smith (2017) and WWF et al. (2012) where fisheries entanglement was identified as the principal cause of mortality in the MRID population, and in the report by Gulland (2009) who concluded that the calves died as a result of interactions with fishing activities.

9.3 Incidental findings

During this study, four incidental findings were identified, that may be adversely affecting this population and, in particular, neonatal survival. Firstly, immunosuppression was likely the principal underlying mechanism facilitating disease emergence in the MRIDs. Secondly, genetic factors, particularly genetic drift and outbreeding depression, were likely affecting this population adversely. Thirdly, aggressive interactions between MRIDs may facilitate opportunistic infections, and finally, as a result of the high neonatal mortality rate, the calving interval of the MRIDs appears shortened. These are discussed in more detail below.

9.3.1 Immunosuppression in the Mekong River Irrawaddy dolphins

This study documented various potential diseases in live and dead MRIDs which were highly suggestive of immunosuppression, including opportunistic infections associated with necrotising fasciitis in calves, and skin lesions detected in dolphins from throughout their geographical range. Necrotising fasciitis has been found in children with underlying immunosuppression (Schro et al., 2019). Immunosuppression was suspected in the current study in the MRIDs, and was also confirmed recently in this population by Schnitzler et al. (2021). Immunosuppression can be influenced by the age/age class, for example human neonates with a developing immune system are highly susceptible to infections (Basha et al., 2014) and aged individuals often have weakened immune systems (Salminen, 2021). In Chapter 4, some necropsied calves were shown to have both omphalitis and lung pathology. These conditions are typical of those expected in immunosuppressed neonatal dolphins (Gulland, 2009). Similarly, in the adult dolphins, lung pathology and infections with opportunistic pathogens implicated immunosuppression in several dead dolphins, with Kuiken et al. (2010 pers. comm.) confirming immunosuppression histologically. Skin lesions

were found in dolphins from all sampled pools (Chapter 6), implying immunosuppression was present at a population level. This may be due to the presence of pathogens/disease, along with anthropogenic contaminants (Repetto and Baliga, 1997; Van Loveren et al., 2000; White et al., 2017).

9.3.1.1 Contaminants and immunosuppression

There is growing evidence for the immuno-toxic effects in dolphins of different environmental contaminants (Beineke et al., 2010; Colborn and Smolen, 2003; de Guise et al., 1998; de Swart et al., 1994; Moeller, 2003; Mori et al., 2008; Mössner and Ballschmiter, 1997; Ross et al., 1996) and in this study several immuno-toxic contaminants at levels sufficient to cause immunosuppression were detected in the sampled dead MRIDs. Chronic exposure to various POPs, such as PCBs, and heavy metals, such as Hg, can predispose dolphins to immunosuppression (Brousseau et al., 2003; Murphy et al., 2009; Schwacke et al., 2012) and result in mortality from infectious diseases (Jepson et al., 1999; Moeller, 2003, Yordy et al., 2010a). In this study, immuno-toxic POP contaminants were found in neonatal MRID calves, presumably from *in utero* and lactational transfer. These contaminants can cause perinatal immunosuppression, resulting in opportunistic infectious diseases, such as necrotising fasciitis. Jepson et al. (1999) found that in harbour porpoises (*P. phocoena*) the most common causes of infectious disease mortality were opportunistic pathogens rather than primary pathogenic agents. This is because opportunistic bacteria normally found in the host or environment generally have low pathogenicity, although can take advantage of compromised host defences to cause disease (Martineau et al., 2003). Jepson et al. (1999) therefore concluded that infections with opportunistic pathogens are consistent with what

might be expected to occur as a result of contaminant-induced immunosuppression, similar to that reported in a study by de Guise et al. (1996) in beluga whales.

9.3.1.2 Immunosuppression and opportunistic pathogens

The POP and Hg levels found in MRIDs in this study were capable of increasing the MRIDs' susceptibility to infectious agents, thus pathogens that have not been documented to cause severe disease in Irrawaddy dolphins may then be more pathogenic in this population. For example, in the study by Carey and Bryant (1995) it was suggested that amphibian mortality does not have to be caused by lethal levels of environmental contaminants. Sub-lethal levels of environmental contaminants, acting singularly or synergistically, could induce stress in young amphibians resulting in immunosuppression, potentially causing fatal infections with opportunistic pathogens, such as *A. hydrophila.* In their study, Carey and Bryant (1995) demonstrated adult frogs survived infections with *A. hydrophila* which were fatal in larval and young metamorphosed frogs. Similarly, neonatal MRID calves may be more vulnerable than adult dolphins to the effects of such opportunistic pathogens, due to their immature immune system, coupled with the deleterious effects of contaminants on their immune function.

Immunosuppression may result in opportunistic infections that may ultimately lead to morbidity or mortality (Cutino-Moguel et al., 2017). The carcasses of 11 beluga whales (*D. leucas*) in the St Lawrence Estuary (SLE) that had been exposed to high contaminant levels and suffered from immunosuppressive diseases, had *A. hydrophila* (n=1), *P. shigelloides* (n=1), *E. tarda* (n=3), and *M. morganii* (n=2) cultured and infection with these organisms were considered to be the primary cause of death (de Guise et al., 2003; Martineau et al.,

2003). Similar opportunistic pathogens were detected in the current study, with the two most frequently cultured opportunistic pathogens being *A. hydrophila* and *P. shigelloides* (Chapters 4 and 5). Whilst *Aeromonas* spp. are ubiquitous in aquatic environments, they have been suggested to be commensals in bottlenose dolphins (*T. truncatus*) (Buck et al., 2006). Furthermore, Buck et al. (1989) demonstrated that *P. shigelloides* and *M. morganii* were also commensals in beluga whales, suggesting that these commensal organisms may be a potential source of infection in immunosuppressed cetaceans, including dolphins. In humans, infections with *P. shigelloides* have been associated with immunosuppression (Janda et al., 2016; Bonatti et al., 2012). Xia et al. (2015) reported that in human neonates with immature immune systems, the case fatality rate for septicaemia from *P. shigelloides* exceeded 50% following perinatal transmission, with mortality usually occurring in the first five days of life. In this study, the culture of opportunistic bacteria from the dead MRIDs, their known presence in healthy cetacean populations, and their known low pathogenicity in healthy hosts, are strongly indicative that the MRIDs are immunosuppressed, similar to that reported by Martineau et al. (2003) in beluga whales.

9.3.2 Genetic factors: genetic drift and outbreeding depression

The finding of I/R alleles in 34% of the dolphins sampled was unexpected and indicates that the population is currently undergoing genetic drift. This was further validated when the number of I/R alleles was compared between adults and calves, demonstrating a lower frequency of I/R alleles in the second generation, similar to that reported by Hoffmann and Willi (2008) in various populations. The reason for the population currently in a state of genetic drift was discussed in Chapter 8, and may be due to the high neonatal mortality rate with no recruitment for several generations, keeping the population genetically static or

frozen in time in a ‘genetic time capsule’. Due to this, the genetic data must be interpreted with caution because an increase in I/R alleles may give the impression of higher than expected genetic diversity (increased heterozygosity) within this population, thereby masking low genetic diversity, and may also mask a bottleneck effect in the population. Additionally, small isolated populations are more likely to be inbred because of a higher probability of mating among close relatives and increased chance of fixation of alleles through genetic drift (Barmentlo et al., 2018). Furthermore, strong genetic drift caused by both founder effects and a bottleneck event could play an important role in causing high genetic differentiation among the MRIDs, similar to that described by Funk et al. (2016) in the island fox (*Urocyon littoralis*).

9.3.2.1 Inbreeding and outbreeding depression

In this study, significant levels of genetic differentiation between coastal and riverine populations and between sampling locations were found. However, Caballero et al. (2018a) found a unique haplotype in *O. brevirostris* from the Gulf of Thailand that was also shared with one *O. brevirostris* from the Mekong River. Thus, it is possible, from an evolutionary perspective, that shared ancestral haplotypes have been maintained in each population.

The excess heterozygosity in this study implies that outbreeding depression is very likely to be occurring in the MRID population. Oakley et al. (2015) described how crosses between natural populations of *Arabidopsis thaliana*, a small flowering plant, can result in heterosis if deleterious mutations have become fixed within the population because of genetic drift. They also stated that divergence between populations could also result in outbreeding depression because of genetic incompatibilities, concluding that the net fitness consequences of between-

population crosses will be a balance between heterosis and outbreeding depression. The findings of increased heterosis in the MRID population may also mask the effects of inbreeding depression in this population.

Outbreeding depression, a phenomenon for which there is very scarce evidence (Edmands, 2007), is thought to be less common than inbreeding depression, and has most commonly been observed in plants and only rarely in animals, however it has been demonstrated in the Arabian oryx (*Oryx leucoryx*) (Marshall and Spalton, 2000), the ornate dragon lizard (*Ctenophorus ornatus*) (LeBas, 2002), Indian rhinoceroses (*Rhinoceros unicornis*) (Zschokke and Baur, 2002), red deer (*Cervus elaphus*), (Coulson et al., 1998), and harbour seals (*Phoca vitulina*) (Coltman et al., 1998). Marshall and Spalton (2000) examined the impact of inbreeding and outbreeding on juvenile survival of reintroduced Arabian oryx from a small number of founding animals from several geographical areas, and noted that the reintroduced population was vulnerable to both inbreeding and outbreeding depression. In their study, they used microsatellite-based measures of inbreeding and outbreeding to show that reintroduced Arabian oryx were affected simultaneously, however they documented the first case where inbreeding depression was masked simultaneously by outbreeding depression. They also found that both very inbred and very outbred calves had low survival rates, and that juvenile survival was also adversely affected (Marshall and Spalton, 2000). They suggested that studies finding no association between inbreeding and fitness should be careful in concluding that inbreeding depression is absent, and that it should become standard practice to test for outbreeding depression as well as inbreeding depression. Thus, it may be possible that the MRIDs may be suffering from both inbreeding and outbreeding depression, however the increased heterozygosity is masking inbreeding depression. Further research is required to validate this hypothesis.

The consequences of outbreeding depression in populations include: high infant mortality and low juvenile survivability, both of which have been demonstrated in the MRIDs. Marshall and Spalton (2000) found that simultaneous inbreeding and outbreeding depression had approximately equivalent effects on juvenile survival in *O. leucoryx* in their studied population. LeBas (2002) also concluded that survivorship in *C. ornatus* was impacted by outbreeding depression, with outbred females having lower reproductive success, as measured by the proportion of their offspring that survived. Similarly, Zschokke and Baur (2002) found higher rates of infant mortality in outbred offspring from Indian rhinoceroses, and survivability was also decreased in largemouth bass (*Micropterus salmoides*) from hybrid individuals (Goldberg et al., 2005). Furthermore, outbred individuals produce offspring with increased susceptibility to infectious diseases. Goldberg et al. (2005) found that outbreeding caused viral resistance to drop by 58% in the F_2 progeny of *M. salmoides*, and that outbreeding resulted in novel infectious diseases emerging in outbred populations. While the data on outbreeding depression are dwarfed by those on inbreeding depression, the few studies that exist suggest the effects can be as detrimental as severe inbreeding (Edmands, 2007).

On-going monitoring of the survival of MRID offspring and long-term population recruitment studies are required, however the results of the studies reported in this thesis would indicate that the MRID offspring currently do not have the functional capacity to survive, and therefore cannot reproduce successfully and add to the population gene pool. Thus, the MRIDs share many similarities to other populations affected by outbreeding depression.

9.3.3 Aggressive interactions

The results presented in this thesis indicate that tooth rakes and puncture wounds are common, with 88% (95%CI 47.3, 99.7) and 94% (95%CI 69.8, 99.8) of cases reported in Chapters 4 and 5, respectively having such skin lesions. Such findings are strongly suggestive of aggression in the MRID population. This has not previously been reported in other Irrawaddy dolphin populations, however were similar to those observed in Risso's dolphins (*G. griseus*) (Mariani et al., 2016).

The reason for the high proportion of tooth rakings and puncture wounds in this population is not clear, however there are two likely explanations based on the studies within this thesis. Firstly, in Chapter 4, dental malformations were diagnosed in two adult dolphins. These dental malformations cause the teeth to protrude horizontally out of the mandible, rather than aligning vertically with the teeth in the maxilla. As these teeth protrude out, they are easily able to inflict tooth rake scars on other dolphins during typical interactions. Further examination of adult MRID skulls collected since 2001, demonstrated that dental malformation appears to be highly prevalent in this population and further research is required to ascertain the extent of such malformations, and whether or not this is the result of a genetically heritable trait which has become fixed over time in the population. Secondly, the traumatic skin lesions seen in this population may be the result of increased aggression in the population. The injuries observed in neonatal calves may indicate infanticidal tendencies in the MRID population, particularly as 80% of calves had puncture wounds around their necks, compared to none in the dead adults. Infanticide may be a strong motive for male dolphins to accelerate a female's return to oestrus and gain sexual access to the mothers (Zheng et al., 2016). This is further supported by the finding that few females in the population appear to be reproducing (Chapter 8). However, infanticidal behaviour is not

restricted to males, as confirmed by Saville (2018 pers. *comm.*) where a captive primiparous bottlenose dolphin (*T. aduncus*) mother demonstrated extreme aggressive and infanticidal behaviour towards her calf, which on necropsy was found to have severe bacterial pneumonia. Maternal infanticide has also been reported in a range of other species, including domestic pigs (*Sus scrofa domesticus*) (Chen et al., 2008), wild moustached tamarins (*Saguinus mystax*) (Culot et al., 2011), royal penguins (*Eudyptes schlegeli*) (St Clair et al., 1995), and black-fronted titi monkeys (*Callicebus nigrifrons*) (Cäsar et al., 2007).

Endocrine disruptors (EDCs) found in the MRIDs, such as DDTs, PCBs, Hg and Cd (Chapter 7), have the potential to interfere with an animal's ability to develop and behave normally, to reproduce, or to cope with stress and infectious disease (Colborn et al., 1993). As a result, this population may be at risk due to the disruption of physiological and behavioural responses to environmental cues (Patisaul and Adewale, 2009). Such behavioural responses are paramount to the reproductive fitness and the stability of a population (Patisaul and Adewale, 2009). For example, a male who responds to a soliciting female with aggression, rather than courtship, has a significantly decreased chance of passing along his genome, even if he is physiologically capable of doing so (Patisaul and Adewale, 2009). If these contaminants are widespread in the environment then it is possible that all males are affected, further reducing the likelihood of the population successfully breeding. Similarly, if hormones are disrupted such that aggressive behaviour results in infanticide, the overall reproductive success of a population may be threatened. For example, a study by Schneider et al. (2003) investigated the effects of progesterone on the parenting behaviour of male mice (*Mus musculus*). The researchers created mice missing the gene that encodes progesterone receptors, so that the mice were no longer affected by progesterone. In their study 74% of male control mice demonstrated aggressive behaviour and committed infanticide, in contrast

none of the mice lacking the progesterone receptors demonstrated aggression or committed infanticide. Moreover, the progesterone deficient mice were more nurturing, frequently touching their pups and returning them to their nests. Chen et al. (2017b) showed that maternal exposure to EDCs, such as PFOA, decreased levels of serum progesterone in Kunming mice (*Mus musculus Km*), thus it is possible that EDCs can disrupt hormones, resulting in aggression in the MRIDs. Furthermore, as approximately equal numbers of dead adult males and females were observed in this study (Chapter 3), then it is unlikely that this aggression is due to an imbalance in the male to female ratio.

9.3.3.1 Implications of traumatic skin lesions

Traumatic skin lesions from either dental malformations or aggressive interactions may serve as entry portals for opportunistic pathogens, as was seen in the MRIDs. This, together with contaminant induced immunosuppression, particularly in the young and the older dolphins, may be sufficient to facilitate fatal infections, such as necrotising fasciitis.

9.3.4 Reduced reproductive success, neonatal mortality and calving intervals.

Using data from Chapter 3, the average number of dead MRID calves detected per year from 2003-2010 was 13 (95%CI 8.4, 24.7), indicating that at the very least 13 calves were born annually. This is threefold proportionally higher than that found by Baker et al. (2018), who reported an average of seven calves born annually in a population of 142 bottlenose dolphins (*T. truncatus*). Based on these numbers it appears that the MRID population, at the time of my studies, was effectively reproducing, as was noted in a report by Smith (2017). Baker et al. (2018) found that the mean annual calving rate for bottlenose dolphins (*T. truncatus*) was 0.29 calves/reproductive female per year, with an 11% mortality rate before one year of age.

Extrapolating the numbers from Baker's et al. (2018) study to the MRIDs (assuming 85 adults, no recruitment in the population, and 50% of the adults are females and are capable of breeding), results in an expected 12 calves produced per year, with an expected mortality of only one calf each year (assuming 11% mortality). The actual results found in this study indicate firstly, a much higher calving rate in this population, and secondly, a significantly higher mortality rate in this population compared to the study of Baker et al. (2018) ($p=0.0002$). However, the paternity results in Chapter 8 imply that few females are actually breeding, and those that are breeding have short inter-calving intervals. This would result in more calves born per reproductive female than would be expected.

The inter-calving interval for MRID or for any other population of *O. brevirostris* is unknown. Baker et al. (2018) reported that bottlenose dolphins have an inter-calving interval between 2.7 ± 0.6 to 3.5 ± 1.3 years. Connor et al. (1996) also reported similar results in bottlenose dolphins. These intervals may be similar to that expected in *O. brevirostris*, as weaning occurs after two years (Owen, 1866; Ross, 2006). However, the death of a calf is likely to shorten the inter-calving interval. For example, in a captive dolphin facility in Australia, a female dolphin (*T. aduncus*) whose calf died shortly after birth went on to produce a healthy calf 18-months later (Saville, 2018 pers.comm). Similarly, Jefferson et al. (2012) also reported 18-month calving intervals in Indo-Pacific humpback dolphins (*S. chinensis*) experiencing high neonatal mortality rates. Zheng et al. (2016) found that the death of a *S. chinensis* calf disrupted the mother's socio-reproductive state, markedly accelerating a female's return to oestrus, and terminating her postpartum anoestrus. This was supported by the studies of Connor et al. (1996) who reported that female dolphins were attractive to males within days of losing a calf, and Mann et al. (2000) who discovered that bottlenose dolphins may become pregnant within two months of losing a newborn calf. This phenomenon occurs

in other species such as cattle, where the female returns to fertile oestrus shortly after the removal of a calf (Hare et al., 2006). In contrast, the inter-calving interval in cattle is lengthened when calves remain with their mother (Neta et al., 2012). In most mammalian species, lactation suppresses fertility (McNeilly, 2001), known as lactational amenorrhoea. Lactational amenorrhoea has been described in humans (Chao, 1987; Howie and McNeilly, 1982; van der Wijden and Manion, 2015; Velasquez et al., 2006) and is hormonally controlled by suckling, which initiates a cascade of hormones, as described by McNeilly (2001) and Chao (1987). However, if suckling is stopped, lactational amenorrhoea is suspended and ovulation resumes (Chao, 1987). Thus, it is probable that when MRID calves die, lactational amenorrhoea is terminated, resulting in a return to oestrus and hence a shortened inter-calving interval.

9.4 Implications for the Mekong River Irrawaddy dolphins

There are several important implications for MRIDs arising from the results of the studies presented in this thesis.

Firstly, factors that cause immunosuppression in the MRIDs pose an ongoing threat to the health and viability of this population, especially as it is already on the verge of extirpation. Failure to mitigate these causal factors will render the MRIDs vulnerable to infections from opportunistic pathogens that can result in the death of newborns as well as older dolphins. Efforts should focus on mitigating the immuno-toxic and endocrine disruptive contaminants identified in this study, particularly as the latter can result in aggression and the MRIDs are susceptible to opportunistic infections and incidental trauma arising from tooth rakes and puncture wounds. The level of immuno-toxic contaminants found in the MRIDs in this study,

together with the finding that the majority of dead calves had neck lesions associated with necrotising fasciitis, indicates that the MRIDs are susceptible to a catastrophic population crash potentially causing extirpation, as explained by de Moura et al. (2012). Disease can play an important role in driving a small population to extirpation (Caughley and Gunn, 1996; Daszak et al., 2000; de Moura et al., 2012; Frick et al., 2010; Gault et al., 2008; Jensen et al., 2011; Kajiwara et al., 2006; Senthilkumar et al., 1999; Spalding and Forrester, 1993; Siebert and Das, 2010; Pedersen et al., 2007). The transition from endemic population persistence to extirpation generally happens gradually in conventional epidemic models (de Moura et al., 2012); however, if host demographics change, extirpation can occur swiftly, with populations suddenly disappearing, even when they had previously persisted at high endemic levels (de Moura et al., 2012).

Secondly, the high number of neonatal deaths demonstrated in this study has rendered the population functionally extinct, as a result of minimal recruitment into the population. Without increasing neonatal survival, this already aged population will reach a point of collapse, where adults will stop reproducing. Evidence of this was found in this study, which should be cause for alarm. If females are no longer capable of reproducing, the population will ultimately die out, resulting in the loss of another unique population.

Finally, the effect of extirpation of this population will result in a significant financial loss for Cambodia as the dolphins are the principal driver of eco-tourism. However, the loss of the MRIDs is an even bigger conservation loss in terms of biodiversity loss, the genetic diversity loss of this unique evolutionary population, and the loss of ecological function as they are the apex predators in the Mekong River. The loss of an apex predator can result in a trophic cascade. This has been shown in Michigan Lake with the loss of largemouth bass (*M.*

salmoides) (Mittelbach et al.,1995), and in Tasmania with the decline of the Tasmanian devils (*Sarcophilus harrisii*) (Hollings et al., 2014; Hollings et al., 2016). Without immediate remedial conservation intervention, this iconic species will be lost forever.

9.5 Implications for future research and policy

The conservation measures needed to successfully mitigate and reverse the excessive mortality in the MRIDs relies on the fundamental understanding of causality driving the population decline. This has been demonstrated in a study by Emery et al. (2021) on the blue-tailed skink (*Cryptoblepharus egeriae*), Lister's gecko (*Lepidodactylus listeria*), the Christmas Island forest skink (*Emoia nativitatis*), and the coastal skink (*Emoia atrocostata*), as well as in a study by Woinarski et al. (2017) in *P. murrayi*, *M. rubicola*, and *E. nativitatis*. The results outlined in this thesis should help guide conservation policy development to urgently mitigate further mortalities, and reduce the current population decline. Failure to do so will result in the extirpation of this population from the Mekong River.

For policy and actions to prevent further dolphin mortality, this study indicates an urgent need to expand focus beyond fishery entanglements to include the role of disease conditions in the MRIDs. Thus, there is a real necessity for on-going disease surveillance and health assessment of the MRID. Specific areas of focus in this population should include: determining the underlying mechanism of immunosuppression and the role that contaminants are contributing, both individually and as combinations; and identifying the exact mechanism of aggression. Further research should also focus on the collection of samples for virological examination as this was lacking in the current study, particularly with reference to skin and neck lesions. Additionally, full health profiles, particularly of mother-calf pairs, would

greatly enhance the understanding of the effects on the immune system and disease progression. Calving history and calving intervals should also be monitored as a component of further photo-identification work. Further studies should also obtain baseline physiological data, with the goal of identifying further health problems in the population, before the population reaches critical levels when it could be too late to initiate a recovery plan, as was the case with the freshwater baiji (*L. vexillifer*) (Reeves and Gales, 2006; Wang et al., 2006; Yang et al., 2006).

The complexity of the immune system and its associated gene complexes make the system particularly sensitive to disruption following outbred events (Goldberg et al., 2005). Future genetic research should include genomic analyses, a new emerging field useful for assessing the immune function of the MRIDs in response to pathogens and immunosuppression. Mapping microsatellite loci on chromosomes in order to evaluate linkage patterns and examine the variation at fitness-related loci, such as the major histocompatibility complex (MHC), should also be undertaken. This is of particular relevance as increased variation of MHC loci has been associated with increased disease resistance, and improved juvenile survival, recruitment and reproductive success (Du et al., 2010; Gillett, 2009; Sepil et al., 2013). Furthermore, gene complexes are also involved in the function of the highly coordinated immune system, therefore their disruption is likely to reduce the efficiency of the immune response (Goldberg et al., 2005).

Relatively little is currently known about changes in gene expression following exposure to various contaminants in Irrawaddy dolphins, and few studies have been conducted regarding contaminant exposure and gene expression in other dolphin species (bottlenose (*T. truncatus*) (Mollenhauer et al., 2009), and Mediterranean stripped dolphins (*Stenella coeruleoalba*)

(Panti et al., 2011)). However, as Frawley et al. (2011) demonstrated, identifying chemical-induced changes in gene expression would contribute to a deeper understanding of the molecular mechanisms of immunotoxicity and would allow for an assessment of the immune system within the confines of traditional toxicology studies. As such this should be a future area of research for the MRIDs. Similarly, transcriptomic profiles using blubber samples of various contaminants, as demonstrated by Mancia et al. (2014), can be used to further investigate the pathophysiology of immuno-toxic contaminants through disrupted molecular pathways, and as such identify genes involved in the activation of an immune response. For example, Mancia et al. (2014) compared the genes in dolphins with the highest PCB levels to those with the lowest PCB concentration and they found that the "gene signatures" were reflective of immune system activation, correlated to PCB exposure. Such further research can be used to facilitate the understanding of the health status of wild dolphins such as the MRIDs.

A comprehensive risk assessment should also be carried out to quantify the risks associated with the various stressors identified in this study. A quantitative risk analysis, comparing each stressor in order to assess a hierarchy of threats, would be a useful guide for developing and prioritising conservation management strategies.

Further studies are also required in diagnosing necrotising fasciitis in live calves, so that early prophylaxis and treatment could be attempted. Although treatment of live wild dolphins has not previously been attempted, and consequently the success rate of such an intervention is unknown, all possible interventions need to be considered because of the critically endangered status of this population.

The results presented in Chapter 3 demonstrated that the restrictions on gillnets reduced mortalities in adults by 5.5%. Further research should focus on additional intervention strategies to reduce the risk of mortality from gillnets to an acceptable level of risk, as currently only one intervention strategy is employed to minimise gillnet entanglement (Limsong et al., 2017). Currently the level of acceptable risk appears high, resulting in unsustainable levels of human induced mortality.

9.6 Recommendations for the Mekong River Irrawaddy dolphins

Based on this study I have formulated a number of recommendations aimed to improve the likelihood of survival of the MRID population:

Firstly, minimising fisheries interaction with dolphins should continue to be a priority; however multiple intervention strategies should be employed to reduce this human-induced mortality in order to preserve the few breeding adults that remain in this population.

Secondly, a short term goal of implementing immediate remedial measures is required to save this population. This should include direct intervention to treat neonates with neck lesions in order to establish some recruitment back into the population. In order to achieve this, an *in situ* dolphin conservation refuge within the Mekong River needs to be established.

Finally, a long-term goal of reducing immuno-toxic environmental contaminants in the Mekong River, and mitigating the effects of environmental contaminants on the MRID population is required. This can be achieved through educational campaigns designed to educate farmers on the toxic effects of POPs to both humans and other animals, including

dolphins, together with sustainable alternative methods for pest-control using non-toxic products or methods, and promotion of the benefits of organic produce. Such produce could be sold at the dolphin-tourism sites to further generate income from eco-tourism, whilst promoting sustainable farming practices. This would be complimentary to the alternative livelihood programs currently undertaken which promote sustainable eco-tourism whilst eliminating gillnet use.

There are also a number of recommendations aimed to enhance the data collection and research on the MRIDS in order to facilitate and document the population recovery. Firstly, it is recommended that further research be directed towards collecting reliable data on anthropogenic stressors, as well as further enhancing mortality research. Secondly, a collaborative network should be established for compiling existing data from all researchers involved with the MRIDs. Finally, current trends in the Mekong River ecosystem should be documented and monitored, particularly with an emphasis on environmental degradation, with the goal of targeting areas for recovery or sustainable conservation management as proposed in this study with *in situ* conservation. However, Siebert and Das (2010) concluded that there is little time remaining to establish appropriate tests for the MRIDs, nor to wait for the opportunity of sampling animals alive or shortly after death. Similarly, Krützen et al. (2018) stressed that immediate and drastic measures are required to reverse the critically grave state of the MRID population.

9.7 Study limitations

There were four important limitations in this study over and above the specific limitations described in each chapter.

Firstly, a key limitation of this study involved working with an endangered population, thus sampling of live animals was not permitted. Although this is difficult, and requires careful planning, the collection of data from live animals is critical to ensure a complete risk assessment can be carried out. This will facilitate comparison between contaminant levels in live dolphins with dead dolphins to quantify the risks in this population, and permit additional measures of risk to be calculated. Furthermore, commensal organisms can be identified in live, presumably healthy MRIDs, and compared with those cultured from dead dolphins. This testing would also permit better genetic data to be collected to assess the relatedness in the population, and assess the level of inbreeding within the population.

Secondly, the case study approach used in several chapters has limitations due to the bias associated with such studies. In particular, they lack the ability to confirm causality. In order to minimise these limitations, the case studies were designed to be as transparent as possible. This was achieved by: describing in detail the data collection and the steps involved in the case selection; explaining the reasoning behind the particular methods chosen; and being explicit about how interpretations and conclusions were reached. However, the strength of the case studies was to contribute more to theory-building and less to theory-testing, by being exploratory and descriptive in nature, thereby demonstrating how individual cases were affected by disease and pathology. This assisted in understanding the neck lesion phenomenon, and provided tentative hypotheses that helped structure the other studies in this thesis. Thus, the limitations of the case studies were offset by situating them within a broader, pluralistic mixed-method research study. Irrespective, the data were sufficiently strong to show that disease was affecting the MRIDs, and in particular that neck lesions were associated with dead calves, and that there were different pathological conditions in dead

dolphins with neck lesions to those without neck lesions. Whilst case-studies have limitations, there was also a strength that came out of the mixed-methodology approach with triangulation by the use of several methods to examine the same phenomenon. For example, the retrospective study in Chapter 3 found that dead calves were significantly more likely to have neck lesions than dead adults. This same finding was supported in the case studies examined in Chapter 4. Triangulation of the data allowed this study to identify aspects of the neck lesion phenomenon more accurately by approaching it from different vantage points using different methods and techniques. Further research using either a cross-sectional study or ideally a census study would strengthen these findings. Census studies have been used in other critically endangered species, such as in the Gilbert's Potoroo (*Potorous gilbertii*) (Vaughan et al. 2009), although it would be challenging to ensure all MRIDs were observed or sampled, given the turbidity of the Mekong River, the elusive nature of the MRIDs, and the difficulties identifying individuals in the field.

Thirdly, environmental pollution and contaminant levels may have altered since the time of collection and analysis of these samples, with Cambodia becoming more developed and urbanised during the last 10 years, potentially resulting in increased and widespread use of environmental contaminants (Jensen et al., 2011; Minh et al., 2006; Wang et al., 2011). Thus, the figures reported in this study are likely to be unrepresentative, and thus potentially conservative. Conversely, if the bans on environmental contaminants were working, these levels may have actually started to decline. However, given the long half-life of POPs (Farlin et al., 2013), this is a slow process, and the MRIDs are, unfortunately, short of time.

Finally, although substantial funding was secured for sample testing in this study, there were insufficient funds to test all samples available. The magnitude of results of this study could be

increased dramatically if sufficient funds were acquired to test all samples that I had collected over the five year period. However, to address this limitation I prioritised animals based firstly on being in CS2 or 3 and secondly with those animals that had neck lesions. Unfortunately, however, this selection process limited the number of "control" dolphins without neck lesions, which should be a priority for further research. Furthermore, these samples were collected over 10 years ago, and circumstances with host, agent and environmental factors may have altered during this time. For instance, increased numbers of dams constructed in the Mekong River may have altered the hydrology, causing habitat fragmentation, changes in fish stocks, and water temperature (Sakaris, 2013). Despite this limitation, data collected on a critically endangered population are still beneficial; however interpretation of the results should be done carefully.

9.8 Conclusions

In conclusion, this study has identified threats associated with mortality in the MRID population, and has recognised a disease of significance, namely necrotising fasciitis, in calves. Existing knowledge from conservation medicine, epidemiology, conservation biology, conservation genetics, wildlife health and human health were combined in this thesis, to understand the aetiology associated with mortality in the MRID population. This study may be enhanced as new knowledge is acquired; however, it serves as a good baseline for guiding recovery efforts for the MRID population. This study demonstrates, firstly, how critical it is to collect baseline monitoring studies in a declining population, and secondly, how studies using a multidisciplinary approach and mixed-methods are effective in identifying threats, so that a holistic understanding of a complex problem can be made. The findings in this study may prove crucial to the survival of the MRIDs, the most critically endangered population of Irrawaddy dolphins in the world.

Whilst calves are still being born, there is some hope that, with the understanding provided by this study, the situation can be turned around. However, if a doomsday clock exists for the MRID, it would be nearing midnight, as time is running out and the hourglass is almost empty.

“So long and thanks for all the fish”

---Douglas Adams---

--1984--

APPENDICES

Appendix 1. Table 1: Summary of Mekong River Irrawaddy dolphin carcasses included in this study

Year	ID #	Specimen number	Included in retrospective analysis	Veterinary necropsy	Necropsy CS 2/3	Included McLellan study	CS 2 /3 included in McLellan	McLellan CS 2 & 3 (Necropsied)	Dove 0= No FI 1=FI 3=CNBD	McLellan 0= No FI 1=FI 3=CNBD	Age group	Carcass condition
2001	1	OBRE01-11/05	1	0	0	0	0	0			Adult	5
	2	OBRE01-12/05	1	0	0	1	1	0	1	1	Adult	2
	3	OBRE01-25/11	1	0	0	1	1	0	3	3	Calf	3
2002	4	OBRE02-08/09	1	0	0	1	1	0	1	0	Calf	2
	5	OBRE02-01/04	1	0	0	0	0	0			Juvenile	5
	6	OBRE02-21/09	1	0	0	1	1	0	1	1	Adult	3
	7	OBRE02-XX/12	1	0	0	1	1	0	1	1	Adult	2
2003	8	OBRE03-29/01	1	0	0	0	0	0			Adult	5
	9	OBRE03-19/02	1	0	0	0	0	0			Calf	5
	10	OBRE03-11/02	1	0	0	0	0	0			Adult	5
	11	OBRE03-25/03	1	0	0	0	0	0			Calf	5
	12	OBRE03-13/03	1	0	0	0	0	0			Calf	4
	13	OBRE03-03/04	1	0	0	0	0	0			Calf	4
	14	OBRE03-10/04	1	0	0	1	0	0	1	3	Adult	4
	15	OBRE03-19/05	1	0	0	0	0	0			Adult	5
	16	OBRE03-11/06	1	0	0	1	1	0	1	3	Adult	2
	17	OBRE03-02/08A	1	0	0	1	1	0	3	3	Adult	2
	18	OBRE03-03/10	1	0	0	0	0	0			Calf	5

Year	ID #	Specimen number	Included in retrospective analysis	Veterinary necropsy	Necropsy CS 2/3	Included McLellan study	CS 2 /3 included in McLellan	McLellan CS 2 & 3 (Necropsied)	Dove 0= No FI 1=FI 3=CNBD	McLellan 0= No FI 1=FI 3=CNBD	Age group	Carcass condition
	19	OBRE03-21/10	1	0	0	0	0	0			Adult	4
	20	OBRE03-01/12	1	0	0	0	0	0			Adult	5
	21	OBRE03-22/12	1	0	0	1	0	0	3	3	Adult	4
2004	22	OBRE04-05/04	1	0	0	0	0	0			Adult	5
	23	OBRE04-28/09	1	1	1	1	1	1	3	0	Calf	2
	24	OBRE04-09/11	1	1	1	1	1	1	3	0	Calf	3
	25	OBRE04-10/11	1	0	0	0	0	0			Adult	4
	26	OBRE04-15/03	1	0	0	0	0	0			Calf	5
	27	OBRE04-24/02	1	0	0	1	1	0	3	0	Calf	3
	28	OBRE04-03/02	1	0	0	1	0	0	3	3	Calf	4
	29	OBRE04-17/02	1	0	0	0	0	0			Adult	5
	30	OBRE04-08/02	1	0	0	0	0	0			Adult	3
	31	OBRE04-13/03	1	0	0	1	0	0	3	1	Adult	4
	32	OBRE04-18/03	1	0	0	1	0	0	3	3	Calf	4
	33	OBRE04-20/03	1	0	0	1	1	0	3	0	Calf	3
	34	OBRE04-22/03	1	0	0	0	0	0			Calf	4
	35	OBRE04-06/06	1	0	0	0	0	0			Adult	2 (parts)
	36	OBRE04-10/06	1	0	0	0	0	0			Calf	5
	37	OBRE04-17/08	1	0	0	0	0	0			Calf	4
	38	OBRE04-22/11	1	1	0	1	0	0	3	3	Calf	4

Year	ID #	Specimen number	Included in retrospective analysis	Veterinary necropsy	Necropsy CS 2/3	Included McLellan study	CS 2 /3 included in McLellan	McLellan CS 2 & 3 (Necropsied)	Dove 0= No FI 1=FI 3=CNBD	McLellan 0= No FI 1=FI 3=CNBD	Age group	Carcass condition
2005	39	OBRE05-16/12	1	0	0	1	1	0	3	0	Calf	2
	40	OBRE05-17/12	1	0	0	1	0	0	1	1	Adult	4
	41	OBRE05-06/01	1	0	0	1	0	0	1	1	Adult	4
	42	OBRE05-20/01	1	1	1	1	1	1	3	3	Calf	3
	43	OBRE05-01/03	1	0	0	1	0	0	3	3	Calf	4
	44	ORBE05-09/03	1	1	1	1	1	1	3	0	Calf	3
	45	ORBE05-18/03	1	1	0	1	0	0	3	3	Calf	4
	46	ORBE05-19/03	1	1	1	1	1	1	3	0	Calf	3
	47	OBRE05-24/05	1	1	0	1	0	0	3	3	Calf	4
	48	OBRE05-10/12	1	1	1	1	1	1	3	3	Calf	3
	49	OBRE05-25/01	1	1	0	0	0	0			Adult	4
	50	OBRE05-18/05	1	0	0	1	0	0	1	3	Adult	4
	51	OBRE05-20/05	1	0	0	0	0	0			Adult	4
	52	OBRE05-XX/12	1	0	0	0	0	0			Calf	5
2006	53	OBRE06-13/01	1	1	1	1	1	1	3	0	Calf	2
	54	OBRE06-03/01	1	0	0	1	0	0	3	3	Calf	4
	55	OBRE06-14/01A	1	1	1	1	1	1	3	0	Calf	3
	56	OBRE06-14/01B	1	1	1	1	1	1	3	0	Calf	3

Year	ID #	Specimen number	Included in retrospective analysis	Veterinary necropsy	Necropsy CS 2/3	Included McLellan study	CS 2 /3 included in McLellan	McLellan CS 2 & 3 (Necropsied)	Dove 0= No FI 1=FI 3=CNBD	McLellan 0= No FI 1=FI 3=CNBD	Age group	Carcass condition
	57	OBRE06-01/02	1	0	0	1	1	0	3	0	Calf	3
	58	OBRE06-03/02	1	0	0	1	0	0	3	0	Calf	4
	59	OBRE06-13/02A	1	0	0	1	0	0	3	3	Calf	4
	60	OBRE06-13/02B	1	1	1	1	1	1	3	0	Calf	2
	61	OBRE06-15/02B	1	0	0	1	0	0	3	0	Calf	4
	62	ORBE06-28/02	1	0	0	1	0	0	3	3	Calf	4
	63	OBRE06-08/01	1	0	0	1	1	0	3	1	Juvenile	3
	64	OBRE06-27-01	1	0	0	0	0	0			Adult	4
	65	OBRE06-15/02A	1	0	0	1	1	0	3	0	Calf	3
	66	OBRE06-22/02	1	0	0	1	0	0	3	3	Calf	4
	67	OBRE06-05/04	1	0	0	1	0	0	3	3	Calf	4
	68	OBRE06-09/06	1	0	0	0	0	0			Adult	4
	69	OBRE06-28/07	1	0	0	1	0	0	3	3	Adult	4
	70	OBRE06-26/11	1	1	1	1	1	1	3	0	Calf	3
	71	OBRE06-09/12	1	1	1	1	1	1	3	0	Calf	2
2007	72	CID 07 001	1	0	0	1	1	0	3	0	Calf	2
	73	CID 07 002	1	1	1	1	1	1	3	3	Calf	2
	74	CID 07 003	1	1	0	0	0	0			Calf	4
	75	CID 07 004	1	1	1	0	0	0			Calf	3

Year	ID #	Specimen number	Included in retrospective analysis	Veterinary necropsy	Necropsy CS 2/3	Included McLellan study	CS 2 /3 included in McLellan	McLellan CS 2 & 3 (Necropsied)	Dove 0= No FI 1=FI 3=CNBD	McLellan 0= No FI 1=FI 3=CNBD	Age group	Carcass condition
	76	CID 07 005	1	1	0	0	0	0			Calf	4
	77	CID 07 006	1	1	0	0	0	0			Calf	4
	78	CID 07 007	1	1	1	1	1	1	0	3	Calf	3
	79	CID 07 008	1	1	0	1	0	0	3	3	Calf	4
	80	CID 07 009	1	1	1	1	1	1	0	3	Calf	2
	81	CID 07 010	1	1	1	1	1	1	0	3	Adult	3
	82	CID 07 011	1	1	1	1	1	1	0	3	Adult	3
	83	CID 07 012	1	1	1	1	1	1	0	0	Calf	2
	84	CID 07 013	1	1	1	1	1	1	3	0	Calf	3
	85	CID 07 014	1	1	1	0	0	0			Calf	2
2008	86	CID 08 002	1	1	1	1	1	1	0	0	Calf	3
	87	CID 08 004	1	1	1	1	1	1	0	3	Adult	3
	88	CID 08 001	1	1	1	1	1	1	0	0	Calf	2
	89	CID 08 003	1	1	1	1	1	1	0	3	Juvenile	3
	90	CID 08 005	1	1	1	1	1	1	0	3	Adult	2
	91	CID 08 006	1	0	0	0	0	0			Juvenile	4
2009	92	CID 09 002	1	1	1	1	1	1	0	0	Adult	2
	93	CID 09 005	1	1	1	1	1	1	3	3	Adult	2
	94	CID 09 001	1	1	1	1	1	1	1	1	Juvenile	3
	95	CID 09 003	1	1	1	1	1	1	0	3	Calf	3
	96	CID 09 008	1	1	1	1	1	1	0	3	Adult	2

Year	ID #	Specimen number	Included in retrospective analysis	Veterinary necropsy	Necropsy CS 2/3	Included McLellan study	CS 2 /3 included in McLellan	McLellan CS 2 & 3 (Necropsied)	Dove 0= No FI 1=FI 3=CNBD	McLellan 0= No FI 1=FI 3=CNBD	Age group	Carcass condition
	97	CID 09 004	1	0	0	0	0	0			Calf	CNBD
	98	CID 09 006	1	0	0	1	1	0	0	3	Juvenile	2
	99	CID 09 007	1	0	0	1	1	0	0	3	Adult	2
	100	CID 09 011	1	0	0	0	0	0			Calf	CNBD
2010	101	CID 10 001	1	1	0	1	0	0	0	1	Calf	4
	102	CID 10 002	1	1	1	1	1	1	0	3	Adult	2
	103	CID 10 005	0	0	0	1	0	0	3	3	CNBD	CNBD
		Total	**102**	**41**	**32**	**69**	**46**	**30**				

Appendix 1. Table 2: Summary of Mekong River Irrawaddy dolphin carcasses included in this study

Year	Dolphin #	Specimen number	Included in Chapter 3	Included in Chapter 4	Included in Chapter 5	Included in Chapter 6	Included in Chapter 7	Included in Chapter 8
	1	OBRE01-11/05	1	0	0	0	0	0
2001	2	OBRE01-12/05	1	0	0	0	0	0
	3	OBRE01-25/11	1	0	0	0	0	0
2002	4	OBRE02-08/09	1	0	0	0	0	1
	5	OBRE02-01/04	1	0	0	0	0	0
	6	OBRE02-21/09	1	0	0	0	0	0
	7	OBRE02-XX/12	1	0	0	0	0	0
2003	8	OBRE03-29/01	1	0	0	0	0	0
	9	OBRE03-19/02	1	0	0	0	0	0
	10	OBRE03-11/02	1	0	0	0	0	0
	11	OBRE03-25/03	1	0	0	0	0	0
	12	OBRE03-13/03	1	0	0	0	0	0
	13	OBRE03-03/04	1	0	0	0	0	0
	14	OBRE03-10/04	1	0	0	0	0	0
	15	OBRE03-19/05	1	0	0	0	0	0
	16	OBRE03-11/06	1	0	0	0	0	0
	17	OBRE03-02/08A	1	0	0	0	0	0
	18	OBRE03-03/10	1	0	0	0	0	0
	19	OBRE03-21/10	1	0	0	0	0	0
	20	OBRE03-01/12	1	0	0	0	0	0
	21	OBRE03-22/12	1	0	0	0	0	0

Year	Dolphin #	Specimen number	Included in Chapter 3	Included in Chapter 4	Included in Chapter 5	Included in Chapter 6	Included in Chapter 7	Included in Chapter 8
2004	22	OBRE04-05/04	1	0	0	0	0	0
	23	OBRE04-28/09	1	0	0	0	0	1
	24	OBRE04-09/11	1	0	0	0	0	1
	25	OBRE04-10/11	1	0	0	0	0	1
	26	OBRE04-15/03	1	0	0	0	0	0
	27	OBRE04-24/02	1	0	0	0	0	0
	28	OBRE04-03/02	1	0	0	0	0	0
	29	OBRE04-17/02	1	0	0	0	0	0
	30	OBRE04-08/02	1	0	0	0	0	0
	31	OBRE04-13/03	1	0	0	0	0	0
	32	OBRE04-18/03	1	0	0	0	0	0
	33	OBRE04-20/03	1	0	0	0	0	0
	34	OBRE04-22/03	1	0	0	0	0	0
	35	OBRE04-06/06	1	0	0	0	0	0
	36	OBRE04-10/06	1	0	0	0	0	0
	37	OBRE04-17/08	1	0	0	0	0	0
	38	OBRE04-22/11	1	0	0	0	0	0
2005	39	OBRE05-16/12	1	0	0	0	1	1
	40	OBRE05-17/12	1	0	0	0	1	1
	41	OBRE05-06/01	1	0	0	0	1	1
	42	OBRE05-20/01	1	0	0	0	0	1
	43	OBRE05-01/03	1	0	0	0	0	1

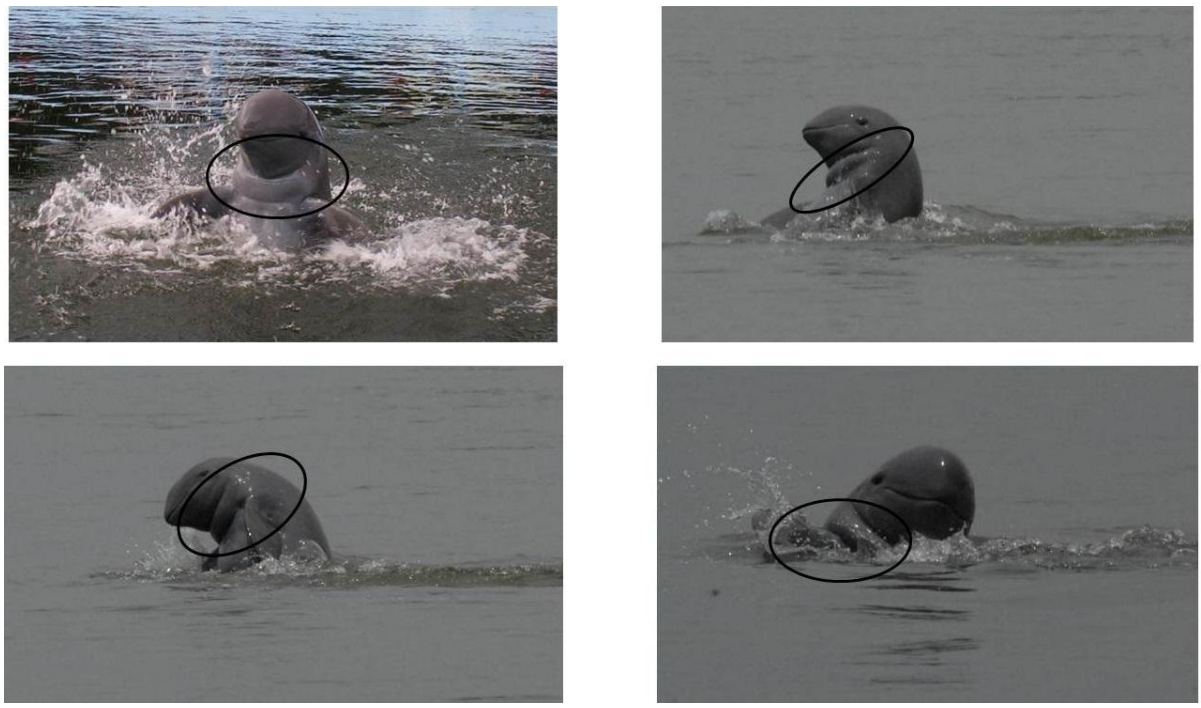
Year	Dolphin #	Specimen number	Included in Chapter 3	Included in Chapter 4	Included in Chapter 5	Included in Chapter 6	Included in Chapter 7	Included in Chapter 8
	44	ORBE05-09/03	1	0	0	0	0	1
	45	ORBE05-18/03	1	0	0	0	0	1
	46	ORBE05-19/03	1	0	0	0	0	1
	47	OBRE05-24/05	1	0	0	0	0	1
	48	OBRE05-10/12	1	0	0	0	0	1
	49	OBRE05-25/01	1	0	0	0	0	1
	50	OBRE05-18/05	1	0	0	0	0	0
	51	OBRE05-20/05	1	0	0	0	0	0
	52	OBRE05-XX/12	1	0	0	0	0	0
2006	53	OBRE06-13/01	1	0	0	0	1	1
	54	OBRE06-03/01	1	0	0	0	0	1
	55	OBRE06-14/01A	1	0	0	0	0	1
	56	OBRE06-14/01B	1	0	0	0	0	1
	57	OBRE06-01/02	1	0	0	0	0	1
	58	OBRE06-03/02	1	0	0	0	0	1
	59	OBRE06-13/02A	1	0	0	0	0	1
	60	OBRE06-13/02B	1	0	0	0	1	1
	61	OBRE06-15/02B	1	0	0	0	0	1
	62	ORBE06-28/02	1	0	0	0	0	1
	63	OBRE06-08/01	1	0	0	0	0	0
	64	OBRE06-27-01	1	0	0	0	0	0
	65	OBRE06-15/02A	1	0	0	0	0	0

Year	Dolphin #	Specimen number	Included in Chapter 3	Included in Chapter 4	Included in Chapter 5	Included in Chapter 6	Included in Chapter 7	Included in Chapter 8
	66	OBRE06-22/02	1	0	0	0	0	0
	67	OBRE06-05/04	1	0	0	0	0	0
	68	OBRE06-09/06	1	0	0	0	0	0
	69	OBRE06-28/07	1	0	0	0	0	0
	70	OBRE06-26/11	1	0	0	0	0	0
	71	OBRE06-09/12	1	1	0	0	1	0
2007	72	CID 07 001	1	0	0	0	0	0
	73	CID 07 002	1	0	0	0	1	0
	74	CID 07 003	1	0	0	0	0	1
	75	CID 07 004	1	0	0	0	0	1
	76	CID 07 005	1	0	0	0	0	0
	77	CID 07 006	1	0	0	0	0	0
	78	CID 07 007	1	0	1	0	0	1
	79	CID 07 008	1	0	0	0	0	0
	80	CID 07 009	1	0	1	1	1	1
	81	CID 07 010	1	0	0	0	1	1
	82	CID 07 011	1	0	1	0	1	1
	83	CID 07 012	1	1	1	0	1	1
	84	CID 07 013	1	0	0	0	1	1
	85	CID 07 014	1	1	1	0	1	1

Year	Dolphin #	Specimen number	Included in Chapter 3	Included in Chapter 4	Included in Chapter 5	Included in Chapter 6	Included in Chapter 7	Included in Chapter 8
2008	86	CID 08 002	1	0	1	0	1	1
	87	CID 08 004	1	0	0	0	0	1
	88	CID 08 001	1	1	1	0	1	1
	89	CID 08 003	1	0	1	0	1	1
	90	CID 08 005	1	0	1	0	1	1
	91	CID 08 006	1	0	0	0	0	
2009	92	CID 09 002	1	1	1	0	1	1
	93	CID 09 005	1	0	0	0	1	1
	94	CID 09 001	1	0	1	0	1	1
	95	CID 09 003	1	0	1	0	1	1
	96	CID 09 008	1	1	1	0	1	1
	97	CID 09 004	1	0	0	0	0	0
	98	CID 09 006	1	0	1	0	0	0
	99	CID 09 007	1	0	0	0	0	0
	100	CID 09 011	1	0	0	0	0	0
2010	101	CID 10 001	1	0	1	0	0	0
	102	CID 10 002	1	1	1	0	0	0
	103	CID 10 005	0	0	0	0	0	0
Total			**102**	**7**	**16**	**1**	**22**	**44**

Appendix 2. Neck fold in Mekong River Irrawaddy dolphins

Neck folds in *O. brevirostris* foetuses *in utero.* (Chapter 4, Case-8).



Neck folds circled, in photographs taken of adult *O. brevirostris* during photo-identification surveys (Chapter 6).

Appendix 3. Necropsy protocol

Orcaella brevirostris

Specimen Number:	CID-Year-ID number
Date of Necropsy:	
Conducted by:	
Assisted By:	
Location of carcass:	
Cause of Death:	
Age:	
Sex:	
Decomposition score:	
Total length:	cm
Weight:	kg

General Comments/ History:

- Dolphin initially recovered from:
- _____
- _____
- _____
- _____
- _____
- GPS Co-ordinates UTM _____48p_____

External Exam:

- Gross findings
- Take Photos
  - □ Skin
  - □ Lesions
  - □ Dorsal fin
  - □ Neck lesion (both sides)

Internal Exam:

Respiratory system:

- Gross findings
- Take Photos
  - □ Lungs
  - □ Blow hole
  - □ Mouth
  - □ Air pipe

Cardiovascular System:

- Gross findings
- Take Photos
  - □ Heart (closed and inside)

Alimentary/ Gastrointestinal tract (Liver, pancreas, SI, LI, colon,):

- Gross findings
- Take photos
  - □ Liver
  - □ Stomach
  - □ Intestines
  - □ Tongue
  - □ Teeth

Haemopoietic system/ Spleen:

- Gross findings
- Take photos
  - □ Spleen found near stomach small black mass

Urogenital system:

- Gross findings
- Take photos
  - □ Bladder inside and outside
  - □ Testicles (male) and penis
  - □ Uterus and ovaries (female)
  - □ Kidneys (both)

Musculoskeletal system:

- Gross findings
- Take photos
  - □ Neck lesion
  - □ All muscle over whole body (after blubber removed) both sides

Nervous system:

- Gross findings
- Take photos
  - □ Brain

Endocrine system:

- Gross findings
- Take photos
  - □ Adrenal glands (found in front of kidneys)

Special Senses (eye/ears):

- Gross findings
- Take photos
  - □ Eyes both
  - □ Ears if visible

Summary of significant findings:

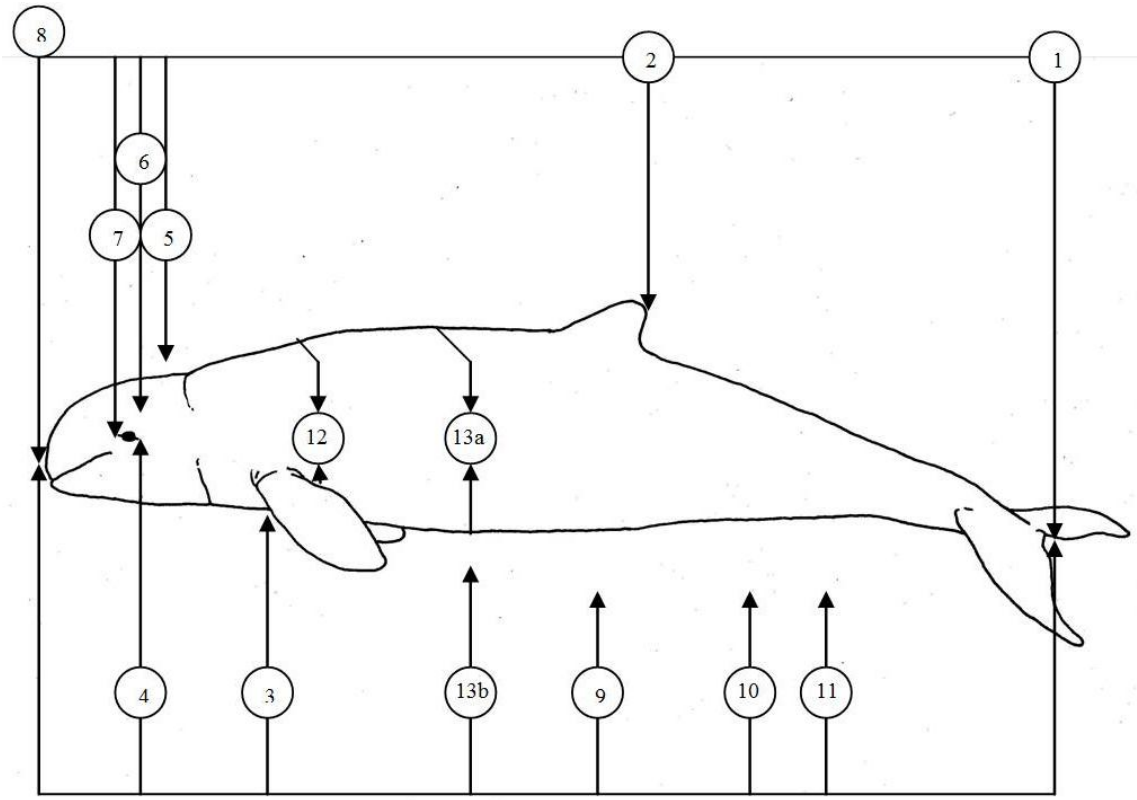
- Major findings:
  - What were the main problems with this dolphin?
  - _____
  - _____
  - _____
  - _____
  - _____
  - _____
  - _____

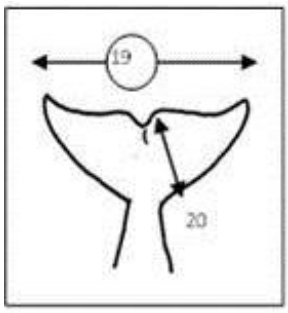
Tissue Samples:

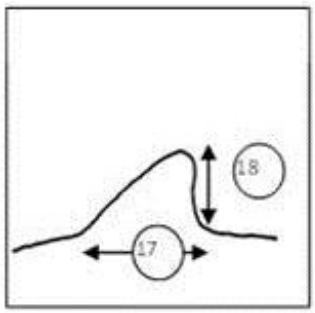
Study	Samples
Formalin tissues:	☐ blubber ☐ tongue ☐ oesophagus ☐ larynx ☐ trachea ☐ lung ☐ heart ☐ aorta ☐ pulmonary artery ☐ liver ☐ pancreas ☐ stomach (3 chambers) ☐ SI (duodenum, ileum) ☐ LI (colon) ☐ uterus ☐ ovaries (1/2) ☐ adrenal glands ☐ kidneys ☐ brain ☐ skin ☐ muscle ☐ diaphragm ☐ melon ☐ spleen ☐ bladder ☐ lymph nodes
Pathology:	Collect anything abnormal examples: ☐ skin lesions ☐ neck lesion ☐ black muscle ☐ lung nodules
Toxicology: plastic tubs	☐ blubber (B, D, E, H) ☐ kidney (left and Right) ☐ liver ☐ melon ☐ muscle ☐ brain
Toxicology: plastic bags:	☐ blubber (D) ☐ kidney ☐ liver ☐ muscle ☐ melon

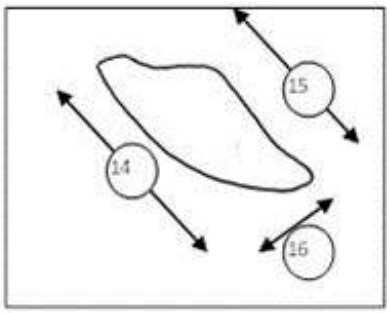
Study	Samples
Toxicology: aluminium foil	☐ blubber (D) ☐ kidney ☐ liver ☐ muscle ☐ melon
Reproduction: plastic bags:	☐ uterus and ovaries (females) ☐ testes (males)
Microbiology (Institute du Pasteur):	Collect the following in a sterile manner, first wash with iodine then alcohol, do not contaminate the samples. Place in sterile container ☐ neck lesion 1x; adjacent muscle 1x ☐ lung ☐ kidney ☐ heart/ aorta ☐ faeces ☐ anything pathological
Cryotubes:	☐ skin
Stomach contents:	☐ if present place in plastic and freeze
Teeth:	☐ collect and freeze
Genetics:	☐ skin frozen in plastic
Skeleton	☐ collect

Irrawaddy Dolphin Morphometrics

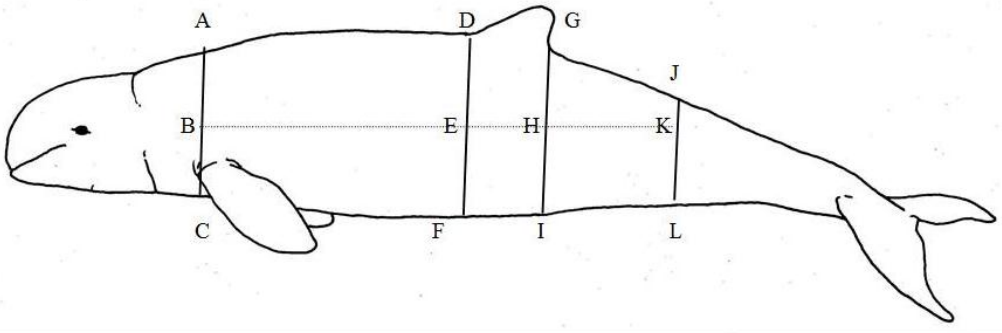








Blubber Measurements in mm



Appendix 4. Necropsy report template

Orcaella brevirostris

Specimen number:	CID-(Year)-(carcass number)
Date of necropsy:	
Conducted by:	Dr Verné Dove
Assisted by:	
Location of carcass:	
Cause of death:	
Age:	
Sex:	
Decomposition score:	
Total length (cm):	
Weight (kgs):	

General comments/ history:

- Dolphin initially recovered from:
- _____
- _____
- _____

External exam:

- _____
- _____

Internal exam:

Respiratory system:

- _____

Cardiovascular system:

- _____

Alimentary/ gastrointestinal tract (liver, pancreas, SI, LI, colon,):

- _____

Haemopoetic system/ spleen:

- _____

Urogenital system:

- _____

Musculoskeletal system:

- _____

Nervous system:

- _____

Endocrine system:

- _____

Special senses (eye/ears):

- _____

<u>Summary of significant findings:</u>

- Major findings:
  - _____

Tissue Samples:

Formalin tissues:	Blubber, tongue, oesophagus, larynx, trachea, lung, heart, aorta, pulmonary artery, liver, pancreas, stomach (3 chambers), SI (duodenum, ileum), LI (colon), uterus, ovaries, testes, adrenal glands, kidneys, brain, skin, muscle, diaphragm, melon, spleen, bladder, LN's,
Pathology:	Pathological tissues:
Toxicology: plastic tubs	Blubber (B, D, E, H), kidney, liver, melon, muscle, brain
Toxicology: plastic bags:	Blubber (D), kidney, liver, muscle, melon
Toxicology: aluminium foil:	Blubber (D), kidney, liver, muscle, melon
Microbiology:	Pathological tissues:
Cryotubes:	Skin
Stomach contents:	None/collected
Teeth:	None/collected
Genetics:	Skin: frozen/alcohol/EDTA
Skeleton	Yes/No

Blubber measurements in mm:

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**Average**
Epidermal Layer													
Blubber Layer													
Total													

Results:

- _____
- _____
- _____

Appendix 5. Microbiological and summary histopathological results for seven cases submitted for tissue analysis (Chapter 4).

Case #	Microorganisms Cultured	Histopathology (Kuiken 2010, pers. comm.)		
		Findings	**Diagnosis**	**Interpretation**
Case 1 OBRE 06 09 12	n/a	Gastric ulcer: The exposed submucosa was infiltrated by moderate numbers of cells, suggesting a pre-existing lesion.	Stomach: multifocal, gastric ulceration,	The stomach ulcers were present before death.
Case 2 CID 07 009	*Aeromonas hydrophila Morganella morganii*	Skin defects: The dermis had a diffuse superficial presence of moderate numbers of cells.	Skin: skin ulceration, multifocal.	
Case 3 CID 07 012	*A. hydrophila Plesiomonas shigelloides*	Lung lesion: Diffusely, the lung tissue, alveoli and bronchi were markedly affected by inflammation (neutrophils and alveolar macrophages) and necrosis. There was diffuse fibrin deposition, including fibrin thrombi within capillaries, as well as congestion. The pulmonary blood vessels were oedematous, with abundant fibrin in the lumina.	Lung: bronchopneumonia, pyogranulomatous, necrotising, diffuse, chronic, marked.	The histological diagnosis was consistent with the results of gross necropsy (diffuse pyogranulomatous pneumonia) and bacteriological findings (*A. hydrophila and P. shigelloides*).
Case 4 CID 07 014	*P. shigelloides*	Skin lesion: medium-sized aggregates of inflammatory cells, with lymphocytes and plasma cells, and few macrophages.	Blubber: panniculitis, multifocal,	The histologic lesions were two foci of chronic inflammation of the blubber. This may be associated with

Case #	Microorganisms Cultured	Histopathology (Kuiken 2010, pers. comm.)		
		Findings	**Diagnosis**	**Interpretation**
			lymphocytic, multifocal, chronic, mild.	the neck wound observed grossly and would be consistent with the inflammatory response to such a wound.
Case 5 CID 08 001	*P. shigelloides*	Larynx: There were abundant lymphocytes around the submucosal glands in the laryngeal wall.	No histological diagnoses were made.	
Case 6 CID 09 002	*A. hydrophila*	Lung: In the lumen of few alveoli and bronchioles were moderate numbers of neutrophils and fewer macrophages, mixed with fibrin and cellular debris. Liver: The hepatocytes in the periportal areas had many variably-sized, well-delimited, round vacuoles (fat) in the cytoplasm. In contrast, the hepatocytes in the periacinar areas are small with dark pink cytoplasm. Spleen: The lymphoid follicles were mildly hypocellular Skin lesions: The exposed dermis is covered by a layer of cell debris mixed with many rods and cocci. Diffusely in the underlying dermis were moderate numbers of neutrophils, underlying dermis had focal haemorrhage Lymph node: In the subcapsular sinuses	Lung: suppurative, multifocal, acute, moderate, bronchopneumonia, Liver: diffuse, moderate, hepatic atrophy, Liver: periportal, moderate, hepatic lipidosis, Skin: multifocal, ulcerative, acute, mild, dermatitis, Kidney: multifocal, mild, Renal tubular mineralization,	The lesions in the lung were most suggestive of a bacterial or viral infection. The lesions in the liver suggest chronic emaciation, and mobilization of lipids from adipose tissue with hepatic lipidosis. The lesions in the skin were consistent with superficial lacerations followed by bacterial infection. The lesions in the kidney were incidental. The black pigment observed in lymph nodes around the lungs was consistent with the morphological diagnosis of anthracosis. The lymph node draining the lungs, showed no lymphoid hyperplasia as expected in reaction to bronchopneumonia. This, together with the hypocellularity of the white pulp of the spleen, suggests that the

Case #	Microorganisms Cultured	Histopathology (Kuiken 2010, pers. comm.)		
		Findings	**Diagnosis**	**Interpretation**
		were moderate numbers of macrophages and neutrophils, indicating inflammation in the drainage area of this lymph node. Scattered throughout the cortex are a few macrophages with black pigment (carbon) in the cytoplasm (anthracosis) Lung: There were small aggregates of variably sized cocci and bacilli on the alveolar walls without associated infiltration of inflammatory cells Lung and bronchus: There were multiple small groups of alveoli, in which the lumina contain moderate numbers of neutrophils and occasional macrophages, mixed with variable proportions of oedema fluid, fibrin and cellular debris. In some of these exudates are small aggregates of variably sized bacilli. Some bronchial lumina contain similar inflammatory exudate.	Lymph node: mild, anthracosis	immune response of this animal was lacking. Taken together, the gross and histologic findings suggest an old animal with dental attrition that was severely emaciated.
Case 7 CID 09 008	*Citrobacter freundii, Klebsiella pneumoniae,* *β*-haemolytic Group D *Streptococcus* spp.,	Skeletal & subcutaneous muscle: myocytes with banana-shaped nucleated blue organisms (bradyzoites). Liver: No abnormality detected (nad). [but ghost of focal inflammatory lesion present]	Brain: leptomeningitis, suppurative, diffuse, acute, severe Skeletal muscle:	The most significant histological finding was the suppurative meningitis. The bradyzoites in the skeletal muscle were indicative of a protozoal infection with *Sarcocystis* species.

Case #	Microorganisms Cultured	Histopathology (Kuiken 2010, pers. comm.)		
		Findings	**Diagnosis**	**Interpretation**
	Enterobacter aerogenes, *Escherichia coli*, non-*albicans Candida* spp., Urine: E*nterobacter cloacae*,	Skin: diffuse epidermal hyperplasia. Lung: In the lumen of some bronchioles and alveoli were variably sized pink round bodies (remains of lungworms?). Testis: About one-third of the tissue appears to consist of fibrous connective tissue. Medulla oblongata: There were a few neutrophils in the pia mater. Pons: There were a few neutrophils in the pia mater. Cerebrum: Diffusely in the pia mater was a moderate number of neutrophils.	Sarcocystis species infection. Skin: epidermal hyperplasia, focal to diffuse, moderate.	

Appendix 6. Histopathological results of dolphins with neck lesions (Chapter 5)

Case #	Gross pathological lesions found at necropsy	Pathological samples submitted for histopathological analysis	Histopathological results provided by Kuiken (2010 pers. comm.)
2 CID 07 009	Neck lesion Tattoo lesions Hepatomegaly Lung congestion	Tattoo skin lesions (X4), puncture lesions on neck with underlying muscle, liver, scratch wound, lung	Tattoo Skin Lesion: Multifocal skin ulceration with possible infiltration of inflammatory cells undefined due to autolysis Liver: Autolysis, no abnormality detected (nad)
4 CID 07 012	Neck lesion Skin lesion Diffuse pyogranulomatous pneumonia Hepatomegaly Petechial & ecchymotic haemorrhages	Lung granulomas, liver, lacerations (tooth rakings, & from right side of head), bruised muscle (ventral neck), petechial haemorrhages on epidermis, ecchymotic haemorrhages, dark areas of discolouration on epidermis	Lung: inflammation (neutrophils) and necrosis visible. Fibrin thrombi, fibrin and congestion noted. Diagnosis: "bronchopneumonia, pyogranulomatous, necrotising, chronic, diffuse, marked". Liver: Multifocal fibrin deposition
5 CID 07 014	Neck lesion Hepatomegaly	Neck lesions, gangrenous muscle, skin lesions	Skin lesion: inflammatory cells (lymphocytes, plasma cells, macrophages): Diagnosis: panniculitis, multifocal, lymphocytic, multifocal, chronic, mild Liver: parenchyma has multiple large round spaces (possible post mortem gas formation).
6 CID 08 001	Neck lesion Hepatomegaly Petechial haemorrhages Plaques on liver	Neck lesion, gangrenous muscle, skin lesions, areas with white foci, petechial haemorrhages.	Larynx: abundant lymphocytes around the submucosal glands in the laryngeal wall. Gangrenous muscle: The muscle fibres are separated by empty spaces (gas formation), many bacilli present.
11 CID 09 002	Dilated Cardiomyopathy Pulmonary oedema	Lung, skin lesions, heart, aorta, pulmonary lymph nodes.	Skin Lesion: neutrophils and focal haemorrhage under dermis Diagnosis: dermatitis, multifocal, ulcerative, acute, mild.

Case #	Gross pathological lesions found at necropsy	Pathological samples submitted for histopathological analysis	Histopathological results provided by Kuiken (2010 pers. comm.)
	Pulmonary masses/lymph nodes Pneumonia Hepatomegaly Emaciated Skin wounds		Lung: neutrophils, macrophages, mixed with fibrin, oedema fluid, and cellular debris in the alveoli and bronchioles. Diagnosis: bronchopneumonia, suppurative, multifocal, acute, moderate. Liver: peri-portal hepatocytes have fat vacuoles in the cytoplasm. Peri-acinar hepatocytes are small with dark pink cytoplasm. Diagnosis: hepatic atrophy, diffuse, moderate. Spleen: hypocellular lymphoid follicles. Lymph node: macrophages and neutrophils present. Diagnosis: anthracosis, mild. Kidney: Mineralised ducts. Diagnosis: Renal tubular mineralisation, multifocal, mild.
14 **CID 09 008**	Testicular Mass Pulmonary Oedema Lymphomegaly Sub-dermal haemorrhage Haemothorax Haemoabdomen Haematuria Dental malformation	Pulmonary lymph nodes, Skin lesion (lacerations and tattoo lesion), testicles, blubber with haemorrhage.	Skin lesion: bradyzoites in the subcutaneous muscle and epidermis diffusely hyperplastic Diagnosis: epidermal hyperplasia, focal to diffuse, moderate. Skeletal muscle: bradyzoites in myocytes, Diagnosis: Sarcocystis species infection. Liver: artefact? “ghost of focal inflammatory lesion present” Lung: In the lumen of some bronchioles and alveoli are variably sized pink round bodies (possible lungworms). Brain: neutrophils in the pia mater. Diagnosis: Brain: leptomeningitis, suppurative, diffuse, acute, severe.

Appendix 7. Organic compounds analysed from blubber samples of Mekong River *Orcaella brevirostris*

Organobrominated compounds	Organochlorine Pesticides	Dioxin-like Compounds
3,3',5,5'--Tetrabrombisphenol A	1,3---	1,2,3,4,6,7,8-
Bromocyclen	Hexachlorbutadien	HpCDD
Decabromdiphenylether,PBDE-209	Aldrin	1,2,3,4,6,7,8-
Heptabromdiphenylether,PBDE-183	Chlordan,cis	HpCDF
Heptabromdiphenylether,PBDE-190	Chlordan,trans	1,2,3,4,7,8,9-
Hexabrombenzol	DDD,o,p'	HpCDF
Hexabrombiphenyl,PBB-153	DDD,p,p'	1,2,3,4,7,8-HxCDD
Hexabromcyclododecan	DDE,o,p'	1,2,3,4,7,8-HxCDF
Hexabromdiphenylether,PBDE-138	DDE,p,p'	1,2,3,6,7,8-HxCDD
Hexabromdiphenylether,PBDE-153	DDT,o,p'	1,2,3,6,7,8-HxCDF
Hexabromdiphenylether,PBDE-154	DDT,p,p'	1,2,3,7,8,9-HxCDD
Octabromdiphenylether,PBDE-203	Dieldrin	1,2,3,7,8,9-HxCDF
Pentabrombiphenyl,PBB-101	Dienochlor	1,2,3,7,8-PeCDD
Pentabromdiphenylether,PBDE-100	Endosulfan, alpha	1,2,3,7,8-PeCDF
Pentabromdiphenylether,PBDE-99	Endosulfan, beta	2,3,4,6,7,8-HxCDF
Summe Octabromdiphenylether	Endosulfansulfat	2,3,4,7,8-PeCDF
Summe Pentabromdiphenylether	Endrin	2,3,7,8-TCDD
Tetrabrombiphenyl,PBB-52	HCH (Summe)	2,3,7,8-TCDF
Tetrabromdiphenylether,PBDE-47	HCH, alpha	OCDD
Tribromdiphenylether,	HCH, beta	OCDF
PBDE-28	HCH, delta	WHO---PCDD/F-
	HCH, epsilon	TEQ 1998(incl.
	HCH, gamma	1/2BG)
	Heptachlor	WHO---PCDD/F-
	Heptachlorepoxid,cis	TEQ
	Heptachlorepoxid,trans	1998
	Hexachlorbenzol	(incl.BG)
	Isodrin	WHO---PCDD/F-
	Methoxychlor	TEQ
	Mirex	1998 (ohne BG)
	Nonachlor,trans	
	Octachlorstyrol	
	Oxychlordan	
	Parlar 26	
	Parlar 50	
	Parlar 62	
PCB101	Di-Butyltin	PFOA
PCB138	Di-Octyltin	PFOS
PCB153	Mono-Butyltin	
PCB180	Mono-Octyltin	
PCB28	Tetra-Butyltin	
PCB52	Tri-Butyltin	
	Tri-Cyclohexyltin	
	Tri-Phenyltin	

Appendix 8. Dioxin TEF values used in this study

Dioxin- Compound	TEF
1,2,3,4,6,7,8---HpCDD	0.1
1,2,3,4,6,7,8---HpCDF	0.01
1,2,3,4,7,8,9---HpCDF	0.01
1,2,3,4,7,8---HxCDD	0.1
1,2,3,4,7,8---HxCDF	0.1
1,2,3,6,7,8---HxCDD	0.1
1,2,3,6,7,8---HxCDF	0.1
1,2,3,7,8,9---HxCDD	0.1
1,2,3,7,8,9---HxCDF	0.1
1,2,3,7,8---PeCDD	1
1,2,3,7,8---PeCDF	0.05
2,3,4,6,7,8---HxCDF	0.1
2,3,4,7,8---PeCDF	0.5
2,3,7,8---TCDD	1
2,3,7,8---TCDF	0.1
OCDD	0.0001
OCDF	0.0001
WHO-PCDF/F -TEQ	1

Appendix 9. Control region sequences used for comparison in this study

Location	Accession number	Number of sequences (bp)	Publication
India	GQ851929.1	475	Jayasankar et al., 2011
	GQ507779.1	479	
	GQ507777.1	479	
	GQ507775.1	481	
	GQ507773.1	462	
	GQ507771.1	479	
	GQ507780.1	479	
	GQ507778.1	479	
	GQ507776.1	479	
	GQ507774.1	476	
	GQ507772.1	479	
Thailand	EU121128.1	444	Caballero et al., 2008
Indonesia	GQ922346.1	401	Beasley et al., 2005
Cambodia, Laos PDR	GQ922342.1		
Thailand, Indonesia	GQ922340.1		
Thailand,	GQ922338.1		
Cambodia	GQ922347.1		
	GQ922341.1		
Thailand, Philippines	GQ922339.1		
Indonesia	GQ922337.1		

Appendix 10. Pool coordinates

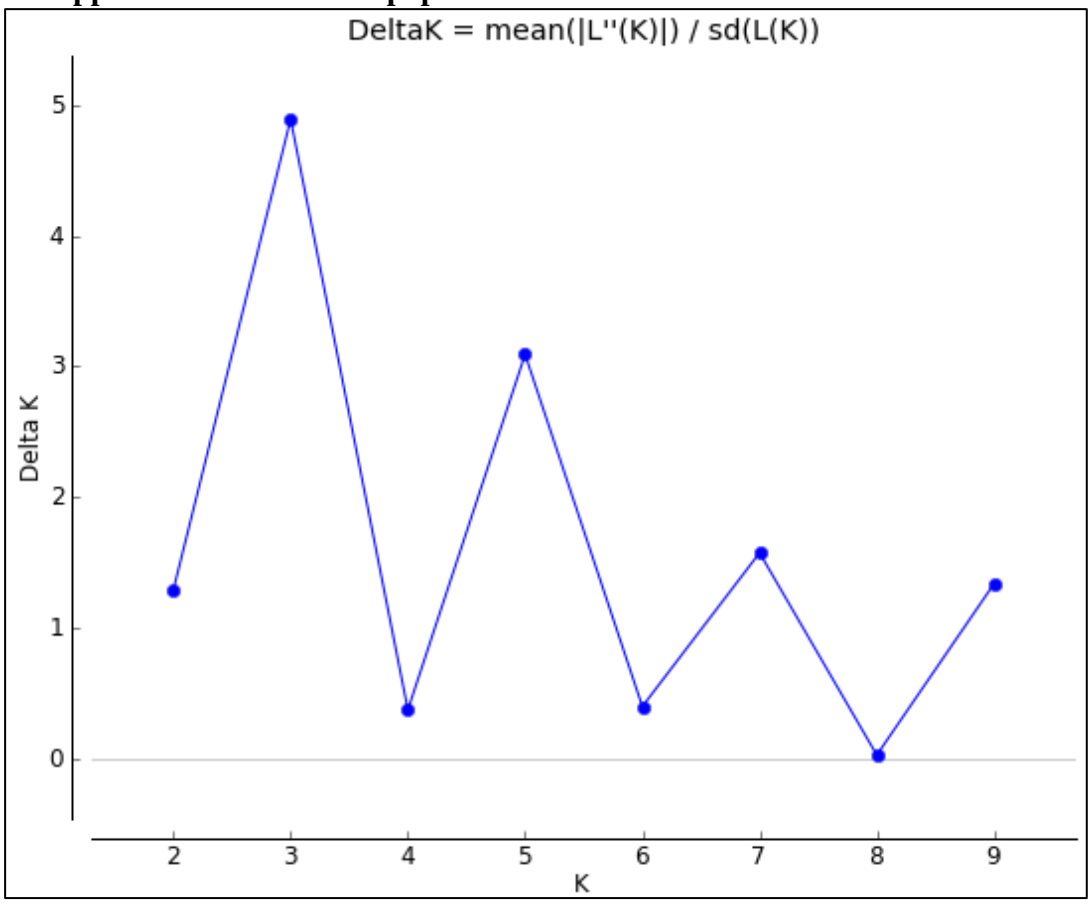
Pool code*	Pool allocation	UTM coordinates	
A	1	<1393131	
1	1	1393131	1394696
B	2	1394696	1411120
2	2	1411120	1412878
C	3	1412878	1418408
3	3	1418408	1420580
D	4	1420580	1431021
4	4	1431021	1434079
E	5	1434079	1456412
5	5	1456412	1457893
F	6	1457893	1464318
6	6	1464318	1467468
G	7	1467468	1472893
7	7	1472893	1474925
H	8	1474925	1480561
8	8	1480561	1482263
I	9	1482263	1539229
9	9	1539229	1539994

*Pool codes in numbers correspond to actual dolphin pools. The letter codes represent the area between pools.

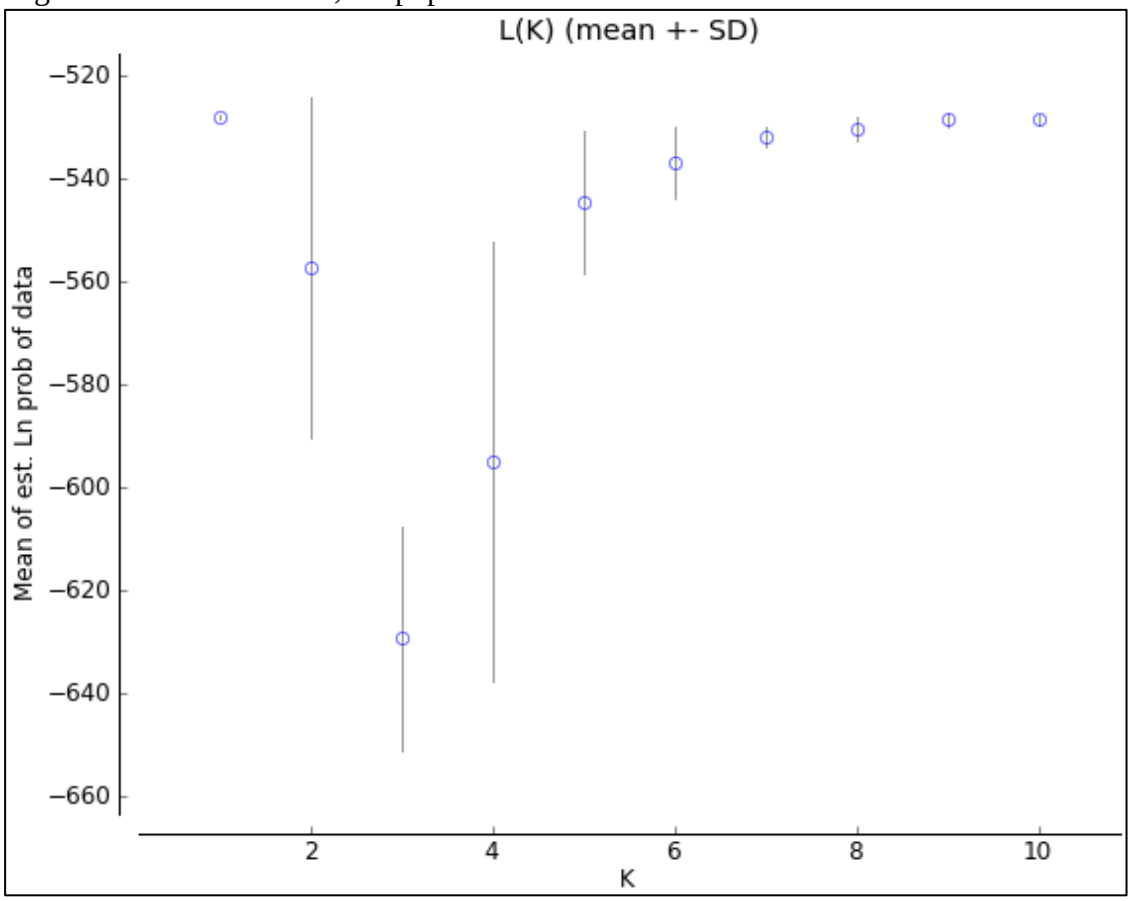
Appendix 11. Null allele analysis

Locus	Null present	Oosterhout	Chakraborty	Brookfield 1	Brookfield 2
GATA98	No	-0.2489	-0.1144	-0.0674	0.3052
EV94	No	0.019	0.0174	0.0152	0.1481
AAT44	No	-0.2728	-0.1828	-0.1559	0.0543
PHO142	No	0.0233	0.0391	0.0215	0.3436
SGUI006	No	0.0972	0.1039	0.08	0.4038
MK6	No	0.0203	0.0462	0.0313	0.2867
SGUI003	No	0.0986	0.1385	0.0991	0.4238
SGUI017	No	-0.0194	-0.0185	-0.0094	0.3758
SGUI016	No	0.1133	0.1287	0.0919	0.4865
SGUI018	Yes	0.3936	0.8682	0.3279	0.5524
SGUI011	Yes	0.3719	1	0.2928	0.7761

Appendix 12. MRID population structure results: Structure harvester



Highest likelihood at *K*=3, for population structure

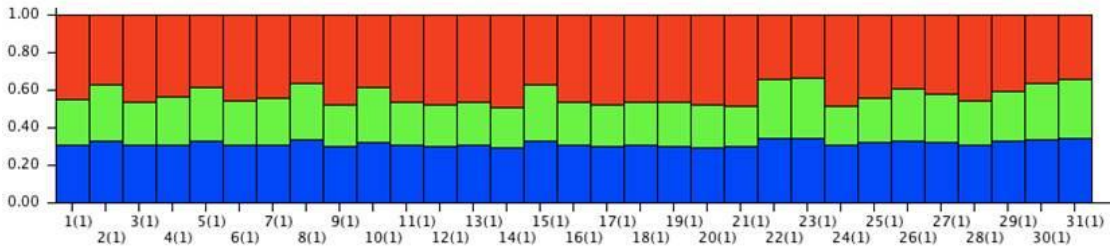


Probability of assignment for population structure

Appendix 13. Evanno output from Structure Harvester

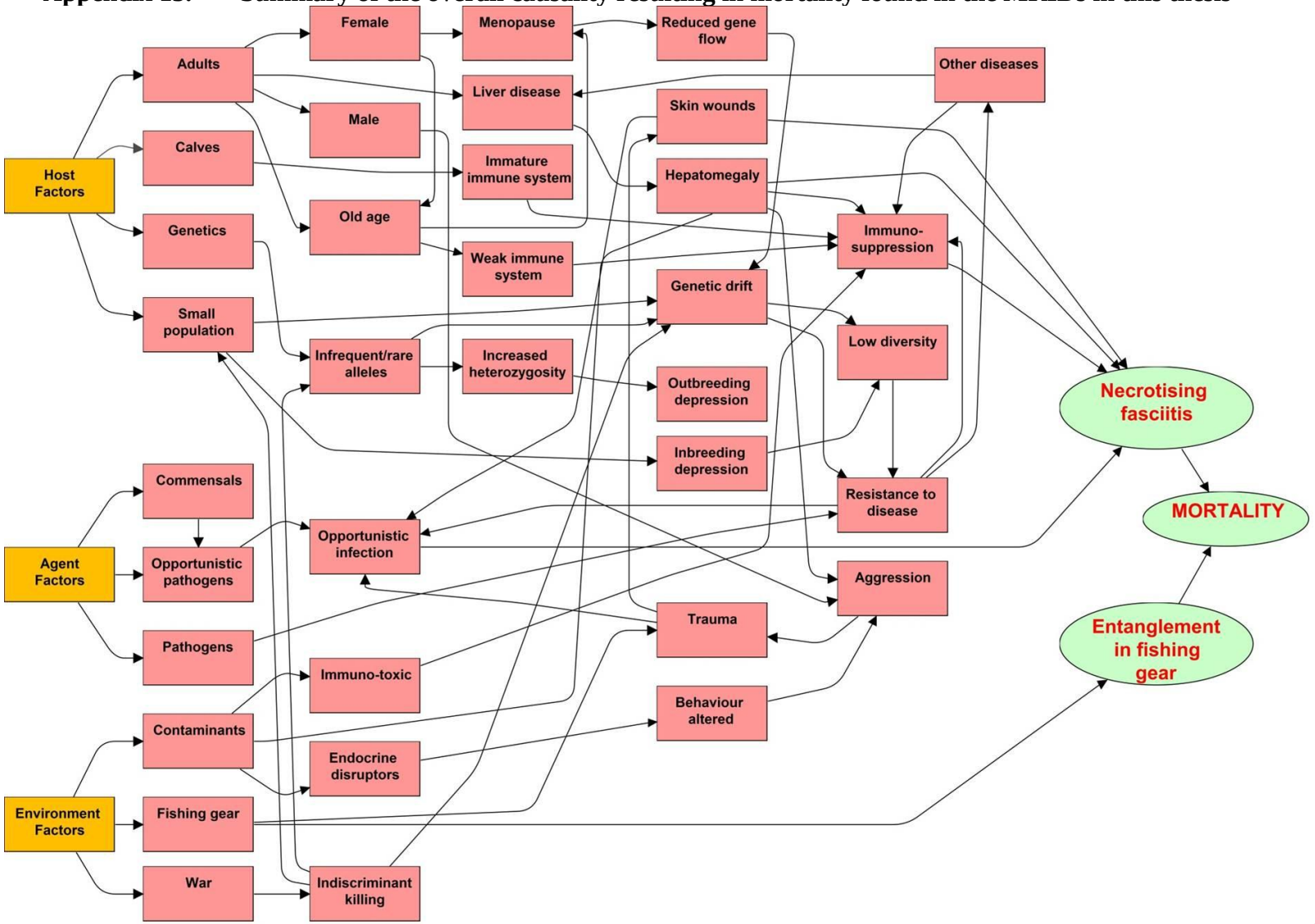
K	Mean LnP(*K*)	Stdev LnP(*K*)	Delta *K*
1	-528.110000	0.381372	—
2	-557.390000	33.005234	1.292522
3	-629.330000	21.760517	4.888671
4	-594.890000	42.735139	0.373229
5	-544.500000	13.832008	3.090658
6	-536.860000	6.998127	0.398678
7	-532.010000	2.040398	1.573223
8	-530.370000	2.169511	0.027656
9	-528.670000	1.182324	1.336352
10	-528.550000	0.913175	—

Appendix 14. Bayesian assignment for each individual inferred by the program structure

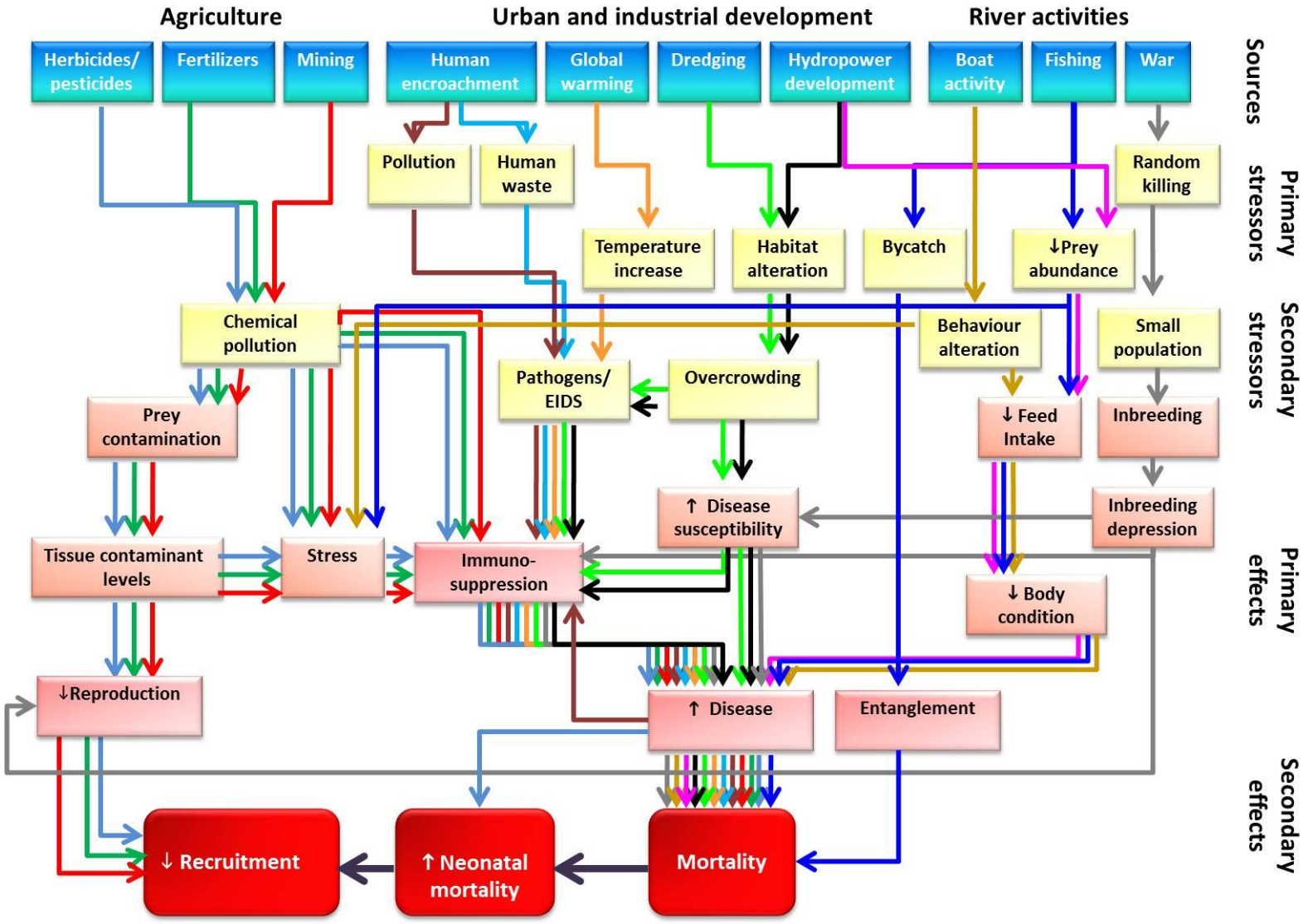


Each colour represents the probability membership coefficient of that individual dolphin to each genetic cluster.

Appendix 15. Summary of the overall causality resulting in mortality found in the MRIDs in this thesis



Appendix 16. Conceptual model of anthropogenic stressors identified in this thesis for Mekong River Irrawaddy dolphins.



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