UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

EVALUATION OF MORBILLIVIRUS AND HERPESVIRUS INFECTION IN CETACEANS STRANDED ALONG THE PORTUGUESE COASTLINE.

MARIA CAROLINA ROCHA DE MEDEIROS BENTO

Orientador(es): Professora Doutora Ana Isabel Simões Pereira Duarte

Professora Doutora Catarina Isabel Costa Simões Eira

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na especialidade de Sanidade Animal

2023

UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

EVALUATION OF MORBILLIVIRUS AND HERPESVIRUS INFECTION IN CETACEANS STRANDED ALONG THE PORTUGUESE COASTLINE.

MARIA CAROLINA ROCHA DE MEDEIROS BENTO

Orientador(es): Professora Doutora Ana Isabel Simões Pereira Duarte

Professora Doutora Catarina Isabel Costa Simões Eira

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na especialidade de Sanidade Animal

Júri

Presidente: Professor Doutor Luís Filipe Lopes da Costa
Vogais: Professor Doutor Mariano Domingo
Professor Doutor Pedro Miguel Amadeu Costa Santos
Professor Doutor Luís Manuel Morgado Tavares
Professora Doutora Ana Isabel Simões Pereira Duarte
Professora Doutora Solange Judite Roque Coelho Alves Gil Neves

2023

DECLARAÇÃO RELATIVA ÀS CONDIÇÕES DE REPRODUÇÃO DA TESE

Nome: Maria Carolina Rocha de Medeiros Bento

Título da Tese ou Dissertação: EVALUATION OF MORBILLIVIRUS AND HERPESVIRUS INFECTION IN CETACEANS STRANDED ALONG THE PORTUGUESE COASTLINE.

Ano de conclusão (indicar o da data da realização das provas públicas): 2023

Designação do curso de Mestrado ou de Doutoramento: Doutoramento em Ciências Veterinárias

Área científica em que melhor se enquadra (assinale uma):

☐ Clínica ☐ Produção Animal e Segurança Alimentar

☐ Morfologia e Função ☒ Sanidade Animal

Declaro sobre compromisso de honra que a tese ou dissertação agora entregue corresponde à que foi aprovada pelo júri constituído pela Faculdade de Medicina Veterinária da ULISBOA.

Declaro que concedo à Faculdade de Medicina Veterinária e aos seus agentes uma licença não-exclusiva para arquivar e tornar acessível, nomeadamente através do seu repositório institucional, nas condições abaixo indicadas, a minha tese ou dissertação, no todo ou em parte, em suporte digital.

Declaro que autorizo a Faculdade de Medicina Veterinária a arquivar mais de uma cópia da tese ou dissertação e a, sem alterar o seu conteúdo, converter o documento entregue, para qualquer formato de ficheiro, meio ou suporte, para efeitos de preservação e acesso.

Retenho todos os direitos de autor relativos à tese ou dissertação, e o direito de a usar em trabalhos futuros (como artigos ou livros).

Concordo que a minha tese ou dissertação seja colocada no repositório da Faculdade de Medicina Veterinária com o seguinte estatuto (assinale um):

1. ☒ Disponibilização imediata do conjunto do trabalho para acesso mundial;
2. ☐ Disponibilização do conjunto do trabalho para acesso exclusivo na Faculdade de Medicina Veterinária durante o período de ☐ 6 meses, ☐ 12 meses, sendo que após o tempo assinalado autorizo o acesso mundial*;

* Indique o motivo do embargo (OBRIGATÓRIO)

Nos exemplares das dissertações de mestrado ou teses de doutoramento entregues para a prestação de provas na Universidade e dos quais é obrigatoriamente enviado um exemplar para depósito na Biblioteca da Faculdade de Medicina Veterinária da Universidade de Lisboa deve constar uma das seguintes declarações (incluir apenas uma das três):

1. É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE/TRABALHO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

Faculdade de Medicina Veterinária da Universidade de Lisboa, 6 de Fevereiro de 2023

Assinatura: Maria Carolina Rocha de Medeiros Bento

À minha família….

Agradecimentos

Ter o privilégio de poder apresentar o meu trabalho para obter o grau de Doutor em Ciências Veterinárias representa o culminar de um esforço pessoal que assentou no apoio incondicional de muitas pessoas a quem agradeço profundamente. Não podia deixar de começar pelo princípio. E o princípio da minha história com os mamíferos marinhos deveu-se ao incentivo e altruísmo de um grupo de pessoas excepcionais. Nesse sentido, começo por agradecer aos amigos de Quiaios, principalmente à Marisa Ferreira, ao José Vingada e à Catarina Eira, que continuou a ter um papel preponderante enquanto minha paciente coorientadora.

Quero agradecer à Professora Ana Duarte, minha orientadora, que esteve presente durante todo o meu percurso na FMV: enquanto professora e enquanto orientadora de mestrado e de doutoramento. Agradeço-lhe do fundo do coração não só todo o apoio técnico e científico mas também as conversas de gabinete e os cafés partilhados que trouxeram leveza e amizade ao meu percurso.

Na FMV tive ainda o apoio e companhia de muitas pessoas, e quero deixar um agradecimento a todas elas, particularmente à Clara que foi uma pessoa que me acompanhou em muitas horas de laboratório e me ajudou bastante e aos alunos de mestrado que partilharam bancada e o seu tempo comigo: o Ricardo, a Inês, o Pedro e tantas outras pessoas que de uma forma ou de outra fizeram parte do meu percurso.

Tenho ainda de agradecer às minhas colegas de trabalho que, ainda que na sua maioria não tenham partilhado no espaço ou no tempo a minha passagem pela FMV, têm sido um pilar fundamental na minha vida por apoiarem em tudo e por me manterem sã e bem disposta. Obrigada Inês, Margarida, Daniela, Raquel, Ana, Carla, Mafalda.

Durante o tempo em que decorreu o meu doutoramento vi partir pessoas importantes e também vi nascer as pessoas mais importantes da minha vida. Resta-me por isso agradecer às minhas filhas Filomena e Helena, por serem a melhor motivação para tudo na vida. Ao Ferdi que me tem sempre incentivado a chegar mais longe e a fazer melhor; aos meus pais, que são um apoio incondicional na minha vida e os melhores avós do mundo, à minha irmã, aos meus padrinhos e por último à minha avó, que partiu antes do final deste meu percurso e a quem devo grande parte do que sou.

Obrigada

Funding

The present work was funded by:

- Project CetSenti (RECI/AAG-GLO/0470/2012) (funded by Fundação Ciência e Tecnologia, I.P.);

- CIISA (UID/CVT/ 00276/2013)

- Project LIFE MarPro LIFE09 NAT/PT/000038 (co-financed by the European Comission)

- Sociedade Portuguesa de Vida Selvagem

AVALIAÇÃO DA INFECÇÃO POR MORBILIVÍRUS E HERPESVÍRUS EM CETÁCEOS ARROJADOS NA COSTA PORTUGUESA.

Resumo

Os arrojamentos de cetáceos são frequentes na costa Portuguesa e representam uma janela de oportunidade única para estudar as doenças e os agentes infecciosos nestas populações de cetáceos. Os morbilivírus revelaram-se particularmente importantes em cetáceos pela sua capacidade de causar doença grave e pela sua alta taxa de mortalidade. Os herpesvírus também são particularmente interessantes por co-evoluirem com o hospedeiro e também por serem capazes de estabelecer latência, passando por períodos de reactivação secundária a episódios de stress imunitário. Foram nossos objectivos investigar a epidemiologia e patofisiologia de morbilivírus e herpesvírus em cetáceos. Além disso, quisemos estabelecer um perfil de expressão de citoquinas em golfinhos comuns saudáveis e testar a possibilidade desse perfil servir como ferramenta para detectar *shifts* imunitários nesta espécie.

Um rastreio molecular realizado para o morbilivírus dos golfinhos revelou que os golfinhos riscados arrojados na costa Portuguesa e Galega têm uma prevalência de cerca de 20% de animais positivos, mais alta que aquela encontrada para golfinhos comuns (1%). Um padrão filogeográfico foi observado na análise filogenética, com sequências virais provenientes das Canárias mais semelhantes às do Mediterrâneo do que as sequências provenientes de Portugal e da Galiza. Infecções por Herpesvirus também foram detectadas (tanto de alfa como gamaherpesvírus). O nosso trabalho detectou herpesvírus em golfinhos comuns pela primeira vez. Detectámos ainda co-infecções com várias estirpes de herpesvírus, bem como com morbilivírus e *Toxoplasma gondii.* A expressão relativa de genes de diferentes citoquinas (IL1β, IL6, TNFα, IL12, IL4,IL10, e IFN-γ). foi também determinada para um grupo de golfinhos comuns saudáveis. Esta expressão relativa tambem foi determinada em dois golfinhos comuns doentes. Os nossos resultados mostraram que em animais saudáveis as citoquina com maior expressão são IL1β, TNFα e IFN-γ, enquanto nos animais doentes houve um aumento de expressão de il-10 com diminuição de expressão de todas as outras citoquinas.

O nosso trabalho clarificou a epidemiologia dos morbilivírus em cetáceos arrojados na costa Portuguesa, contribuiu para a variabilidade das sequências de herpesvírus nas subfamílias de alfa e gamaherpesvírus e suporta a hipótese de utilizar o perfil de citoquinas como uma ferramenta para detectar shifts imunitários em golfinhos arrojados.

Palavras chave: Cetáceos, morbilivírus, herpesvírus, rastreios, citoquinas.

EVALUATION OF MORBILLIVIRUS AND HERPESVIRUS INFECTION IN CETACEANS STRANDED ALONG THE PORTUGUESE COASTLINE.

Abstract

Cetacean strandings are frequent along the Portuguese coastline and stranded specimens provide a unique opportunity to study disease and infectious disease agents in cetacean populations. Morbillivirus is particularly important in cetaceans for its ability to cause disease and for its high mortality rates. Herpesviruses are also particularly interesting since they are known to have close co-evolutionary paths with their hosts and for establishing latent infections that can resurge with any stressors of the immune system. This work aimed at investigating the epidemiology and pathophysiology of cetacean morbillivirus and herpesvirus. Furthermore, we aimed at establishing a profile of cytokine genes expression in healthy common dolphins and evaluate the possibility of using that tool to assess immunological shifts in this species.

A molecular survey for dolphin morbillivirus revealed striped dolphins stranded in the Portuguese and Galician coasts have a prevalence of approximately 20% of positive individuals, higher than prevalence found for common dolphins (1%). A phylogeographic pattern was detected in the phylogenetic analysis with viral sequences from the Canary Islands more similar to those from the Mediterranean than samples from Portugal and Galicia. Herpesvirus infection was also detected in cetaceans, and both alpha and gammaherpesvirus were detected. Our work reported infection by herpesvirus in common dolphins for the first time. Co-infection with different strains of herpesvirus was detected, as well as co-infection with morbillivirus and *Toxoplasma gondii*. Relative expression of cytokine genes (IL1β, IL6, TNFα, IL12, IL4,IL10, and IFN-γ) was also determined for a group of healthy common dolphins. Relative expression was also determined in two sick common dolphins. Our results showed that IL1β, TNFα and IFN-γ were the cytokines with higher relative expression in healthy individuals and that Il-10 was the most expressed gene in the two sick animals, with a downregulation of all other cytokines.

This work clarified the epidemiology of morbillivirus in cetacean species stranded along the Portuguse coastline, contributed to the variability of herpesvirus sequences in the gamma and alphaherpesvirus subfamilies and supports the evidence that profiling cytokine markers may serve as a tool to assess immunological shifts in stranded cetaceans.

Keywords: *Cetacea*, mobillivirus, herpesvirus, surveys, cytokines.

AVALIAÇÃO DA INFECÇÃO POR MORBILIVÍRUS E HERPESVÍRUS EM CETÁCEOS ARROJADOS NA COSTA PORTUGUESA

Resumo alargado

Os arrojamentos de cetáceos são frequentes na costa Portuguesa, não só pela sua extensão costeira mas também por diversos processos oceanográficos qu favorecem esses eventos. Os cetáceos são relativamente abundantes e a captura acidental em artes de pesca representa uma causa de morte importante. Embora a maioria destes arrojamentos envolvam golfinhos comuns (*Delphinus delphis*), que são a espécie mais abundante na costa de Portugal continental, ja foram detectadas 28 espécies incluindo golfinhos riscados (*Stenella coeruleoalba*), bôtos (*Phocoena phocoena*), roazes (*Tursiops truncatus*), cachalotes pigmeu (*Kogia breviceps*), baleias anãs (*Balaenoptera acutororostrata*) e baleias comuns (*Balaenoptera physalus*), entre muitas outras espécies. A realização de protocolos completos de necrópsia com recolha de amostras para virologia, microbiologia, histopatologia, entre outras, que são armazenadas um banco de tecidos, possibiita a realização de estudos retrospectivos nestes animais para determinar, não só a causa de morte, mas para estudar a presença de agentes patogénicos nestas populações.

Os cetáceos arrojados representam uma oportunidade única para estudar as doenças e agentes infecciosos presentes nas suas populações. O morbilivírus dos cetáceos (CeMV) desempenha um papel importante no estatuto sanitário destas populações e vários estudos têm vindo a investigar a sua prevalência e a capacidade de causar doença grave e mortalidades elevadas nestes animais, particularmente em espécies como o golfinho riscado. Outros vírus podem ser relevantes em cetáceos, sendo que os herpesvírus são, por vários motivos, particularmente interessantes: são reconhecidos pelo seu longo processo de co-evolução com as espécies hospedeiras, e têm a capacidade de estabelecer latência, podendo haver reactivação da infecção em episódios de stress imunitário.

Este trabalho teve como objectivos gerais investigar a epidemiologia do morbilivírus nos cetáceos arrojados na costa Portuguesa e Galega, bem como estudar as suas relações filogenéticas com outros vírus amplificados a partir de amostras de cetáceos, com particular ênfase para os encontrados no Mediterrâneo. Outro objectivo foi o rastreio molecular de herpesvírus perceber a prevalência nestas espécies, bem como compreender a fisiopatologia e a relevância deste vírus na saúde destas espécies marinhas. Neste trabalho debrucei-me ainda sobre a expressão de mediaores imunitários para ir de encontro ao objectivo de construir uma ferramenta que permitisse detectar shifts imunológicos em golfinhos comuns. Para isso foram integrados resultados das observações macroscópicas

durante as necrópsias, de histologia e de rastreios moleculares para este vírus (com subsequente análise filogenética), de forma a permitir retirar informações acerca da epidemiologia e patofisiologia destas doenças e foi ainda estabelecido um grupo de golfinhos comuns considerados saudáveis que permitiu estabelecer um perfil de citoquinas relevantes na resposta imunitária.

Para o rastreio molecular de morbilivírus foram testados 279 animais pertencentes a 5 espécies de cetáceos. As duas espécies mais testadas foram os golfinhos comuns (n=193) e os golfinhos riscados (n=69). Nestes últimos, foram testadas amostras provenientes da costa Portuguesa (n=36) e da Galiza (n=33). Observámos uma prevalência total de 5.7% de animais positivos. Estes positivos foram detectados apenas em duas espécies: golfinho comum e golfinho riscado. Relativamente ao mapeamento da infecção detectámos vários golfinhos riscados com positividade a DMV apenas no sistema nervoso central e alguns com positividade no pulmão e noutros órgãos. Nos golfinhos comuns, um dos animais foi positivo em todos os órgãs testados e outro foi positivo em linfonodo pulmonar, pulmão e linfonodo mesentérico. A prevalência em golfinhos riscados (20,3%) revelou-se superior à de golfinhos comuns (1%), sendo essa diferença estatisticamente significativa. A análise filogenética revelou um padrão filogeográfico que parece indicar uma maior proximidade entre as estirpes que circulam no atlântico que banha Portugal e Galiza, enquanto as estirpes do Mediterrâneo se mostrarm mais próximas das detectadas nas ilhas Canárias, o que pode sugerir que estas duas populações são relativamente isoladas umas das outras. Neste sentido, parece que a hipótese mais provável será que as introduções de morbilivírus no Mediterrâneo aconteçam mais frequentemente através da circulação de animais que habitam áreas Atlânticas mais a sul.

Relativamente ao rastreio de herpesvírus, realizou-se um rastreio molecular em 179 animais pertencentes a 6 espécies. Foi detectado herpesvírus em 14 animais (2 bôtos, 2 golfinhos riscados e 10 golfinhos comuns). A condição de conservação de carcaça mostrou-se relevante para acapacidade de detecção de animas positivos, e a prevalência de infecção em animais frescos (códigos 1 e 2 num score de 5) foi de 14,3%. A análise filogenética demosntrou que os herpesvírus detectados pertencem às subfamíias alfaherpesvírus e gamaherpesvírus, não se tendo detectado nenhuma sequência de betaherpesvírus. Dentro dos alfaherpesvírus as sequências de cetáceos formam 3 subgrupos (alfa1, alfa2 e alfa3). Estes alfaherpesvírus formam três grupos distintos diferentes dos géneros previamente descritos mas aparentemente mais próximos dos varicellovírus. Foram detectados animais positivos sem sinais de infecção sistémica por herpesvírus e foram também detectados animais com doença sistémica atribuível a herpesvírus, sendo que além de co-infecção com outros agentes infecciosos (DMV e *Toxoplasma gondii*), detectámos também co-infecção com diferentes herpesvírus no mesmo animal. Amplificação de outros fragmentos

genómicos permitiria uma análise filogenética mais robusta, que não é possível apenas com a amplificação de um fragmento da DNA polimerase que se obteve neste estudo, que ainda assim permite deterimar a subfamília destes vírus.

No que diz respeito ao perfil de citoquinas, conseguirmos estabelecer um grupo de 29 golfinhos comuns considerados saudáveis: animais com captura acidental comprovada em arte xávega que tinham sinais de alimentação recente, boa condição corporal, baixa carga de parasitas e sem sinais de doença na necrópsia. Neste grupo estabelecemos um perfil de expressão relativa de citoquinas no linfonodo pulmonar, nomeadamente: IL1β, IL6, TNFα, IL12, IL4,IL10, e IFN-γ. A expressão relativa destes genes foi normalizado para o gene de referência seleccionado (RPL7). Os dois golfinhos comuns positivos a morbilivírus foram também testados para aferir a possibilidade de utilizar este perfil como uma ferramente para detecção de shifts imunológicos. Os resultados indicam que no grupo de animais saudáveis as citoquinas com maior expressão relativa foram IL1β, TNFα e IFN-γ enquanto as menos expressas foram IL6, IL12, IL4 e IL10. Nos dois animais doentes o que pudémos observar foi uma expressão relativa de il10 superior a qualquer outra citoquina, sendo que quando comparado com o grupo controlo parece haver uma subexpressão de IL1β, TNFα, IFN-γ e IL4. Estes resultados nos dois animais doentes suportam a ideia de terem sucumbido a uma forma crónica de morbilivírus: não se detectou positividade na imunohistoquímica para morbilivírus em nenhum destes dois animais nos órgãos positivos por qPCR, não se detectaram na histopatologia lesões atribuíveis a morbilivírus, os dois animais apresentavam uma toxoplasmose activa que é indicativo também de uma possivel imunosupressão. A sobreexpressão de IL10, uma citoquina anti-inflamatória, é outro factor indicativo da forte imunossupressão nestes animais, que apresentavam na histopatologia uma forte depleção linfóide nos linfonodos, com deposição de substância amilóide.

Este trabalho ajudou a compreender a epidemiologia da infecção por morbilivírus em cetáceos arrojados na costa Portuguesa e Galega. Ficámos com informações relativas não só à prevalência em diferentes espécies mas também acerca do curso da doença e da filogenia dos vírus encontrados. Serviu ainda para aumentar a variabilidade de herpesvírus nos cetáceos e esclarecer as suas relações filogenéticas, incluindo-os nas subfamílias alfaherpesvírus e gamaherpesvírus. Foi ainda possível compreender que os herpesvírus são capazes de causar doença sistémica e ser motivo de morte e arrojamento, ocorrendo também em simultâneo com outros agentes infecciosos como os morbilivírus. Neste trabalho detectou-se pela primeira vez herpesvírus em golfinhos comuns, tendo sido detectados tanto alfa como gamaherpesvírus. Foi ainda possível testar a elaboração de um perfil de citoquinas em golfinhos comuns como uma ferramenta de detecção de shifts imunológicos em animais selvagens.

A utilização de animais arrojados para estudos epidemiológicos é passível de crítica pela *bias* óbvio nestas circunstâncias. Apesar do cuidado que devemos ter ao inferir conclusões destes resultados, eles representam uma oportunidade única para obtermos informações acerca destas populações de vida livre, proporcionando material que de outra forma não conseguiríamos obter. A grande proporção de animais capturados acidentalmente em artes de pesca é também uma forma de diminuir o *bias,* randomizando estas amostras.

A continuidade destes trabalhos é importante para a monitorização de doenças infecciosas nestas populações, idealmente alargando o leque de agentes infecciosos rastreados. A realização de mais estudos na área da imunologia nos cetáceos é relevante, particularmente para compreender a diferente susceptibilidade a diferentes agentes etiológicos nas várias espécies de cetáceos, particularmente no que diz respeito aos morbilivírus em golfinhos riscados.

Table of Contents

List of Figures

List of Tables

List of Abreviations

CeMV – Cetacean morbilliirus

EEZ – exclusive economic zone

RAMM – Rede de Arrojamentos de Mamíferos Marinhos [National Stranding Network]

ICNF – Instituto para a Conservação da Natureza e Florestas

NE-Atlantic – North-east Atlantic

MATB – Marine animal tissue bank

UA – Universidade de Aveiro

SPVS – Sociedade Portiuguesa de Vida Selvagem [Portuguese Wildlife Society]

CITES - Convention on International Trade in Endangered Species of Wild Fauna and Flora

FCT – Fundação para a Ciência e Tecnologia

FMV-UL – Faculdade de Medicina Veterinária da Universidade de Lisboa

UM – Universidade do Minho

HV – Herpesvirus

FeMV – Feline morbillivirus

SSPE – Subacute sclerosing panencephalitis

ODE – Old dog encephalitis

RNP complex – ribonucleoprotein complex

MV – Measles

PMV – Phocine morbillivirus

RPV – Rinderpest

PPRV – Peste des petits ruminants

CDV – Canine Distemper virus

SLAM – Signaling lymphocytic activation molecule

PWMV – Pilot whale morbillivirus

PMV – porpoise morbillivirus

IUCN - International Union for Conservation of Nature

RT-PCR – reverse transcription – PCR

CMV – cytomegalovirus

PhHV – Phocid herpesvirus

DNApol – DNA polymerase

OtHV1 – Otarine herpesvirus1

IFN – interferon

IL – interleukins

TGF – transforming growth factor

NK – natural killer

PP – posterior probability

CEMMA – Coordinadora para o Estudo dos Mamiferos Mariños

DD – *Delphinus delphis*

SC – *Stenella coeruleoalba*

TT – *Tursiops truncates*

GM – *Globicephala melas*

KB – *Kogia breviceps*

MMi – *Mesoplodon mirus*

BP – *Balaenoptera physalus*

NCBI – National Center for Biotechnology Information

PCB – polychlorinated biphenyl

CHAPTER I

INTRODUCTION

1. The Portuguese Scenario on stranding events: sample and data collection

As a maritime country, the ocean is a fundamental and formative element of the Portuguese identity. Having one of the largest Exclusive Economic Zones (EEZ), Portugal has always had a close relationship with the sea. Portugal's EEZ has approximately 1.700 thousand square kilometers, and is one of the world's largest, with a great diversity of maritime ecosystems and resources that can establish Portugal as a model for economic growth in conjunction with marine biodiversity conservation (Resolução do Conselho de ministros 2021).

Portugal has historic traditions in fisheries and Portuguese fisheries represent a primary sector of significant socio-economic importance, particularly in coastal areas. In mainland Portugal, interactions between fisheries and cetaceans and seabirds occur with almost all fishing gears with negative consequences for fisheries and for the conservation status of the accidentally captured species (Vingada et al. 2011).

In the seventies, Teixeira et al. (1979) classified the information on cetaceans in Portugal as mostly unknown; more than 20 years later, the information concerning cetaceans contained in the Red Book of Vertebrates in Portugal is still scarce with a large number of species signaled as *data deficient* (Cabral et al. 2006). The first cetacean abundance estimates in Portuguese continental waters were obtained during the LIFE+ MarPro project. After a compilation of direct observations and stranded individuals, the list of cetaceans occurring in Portuguese waters increased to 28 species (21 Odontoceti species and 7 Mysticeti species) (Vingada and Eira 2017). It was also possible to confirm the occurrence of 5 species that had never been recorded in mainland Portuguese waters: dwarf sperm whale (*Kogia sima*), Sowerby's beaked whale (*Mesoplodon bidens*), True's beaked whale (*Mesoplodon mirus*), Fraser's dolphin (*Lagenodelphis hosei*) and white beaked dolphin (*Lagenorhynchus albirostris*) (Ferreira et al. 2016).

Since 1977 a systematic registry of marine mammals' stranding events is compiled to the National Stranding Network (RAMM) at the Institute for Nature Conservation and Forests (ICNF) (Sequeira et al 1992), the National Authority responsible for wildlife conservation. The decree law 263/81 refers the importance of marine mammals in the marine ecosystems and food chains and highlights the need to adopt protection measures due to the decrease in population numbers of some species. This decree law also prohibits the intentional capture and transport of these species. The transposition of the Habitats Directive to the Portuguese legislation also ensures the protection of cetacean species in Portuguese waters (decree law 140/99 replaced by decree law 49/2005).

Knowledge on marine species is usually limited when compared to the available knowledge on terrestrial species and it is often obtained from stranded animals (Reid et al. 2003). Stranding events are an opportunity window to obtain important information on these species on a myriad of areas: biometrics and anatomy, genetics, toxicology, parasitology, pathology and many more. To understand how infectious diseases and other selection pressures affect cetaceans, studies on ecology and distribution are fundamental, particularly with respect to the most frequently stranded species. Records show that between 2000 and 2012, the Minke whale (*Balaenoptera acuturorostrata*) was the most frequent stranded Mysticeti species (n=32/47) (Figure 1). In the same period, considering only Odontoceti cetaceans, a total of 1177 animals were found stranded and common dolphins (*Delphinus delphis*) were the more commonly found (n=798/1177) followed by porpoises (*Phocoena phocoena*), with 158 individuals. Striped dolphins (*Stenella coeruleoalba*) and bottlenose dolphins (*Tursiops truncatus*) only accounted for 3,8 and 2,3% of all stranded Odontoceti (Ferreira et al. 2016).

Figure 1 - Newborn minke whale and common dolphins stranded on the western coast of Portugal.

Striped dolphins are usually found in temperate, subtropical and tropical waters with a cosmopolitan distribution. They are mostly pelagic and travel in groups of several hundreds or even thousands of individuals (Reyes 1991). In the North-east Atlantic (NE-Atlantic) they are usually found in deep waters (over the 1000-meter bathymetric), outside of the continental shelf (Perrin et al. 1994). Analyses of mitochondrial DNA suggest that populations from the NE-Atlantic are distinct from those in the Mediterranean (García-Martínez et al. 1999). Furthermore, there are also differences in body size between populations from the north Atlantic and those in the Mediterranean (Rice 1998). Bourret et al.

(2007) confirmed significant differences between Mediterranean and Atlantic populations based on the analysis of 5 microsatellites. This is the most common species found in the West and Central Mediterranean (Perrin et al. 1994).

The common dolphin is widely but discontinuously distributed in warm temperate and tropical waters of the Atlantic and Pacific oceans but most areas of distribution coincide with moderate to strong upwelling, and common dolphins generally appear to avoid warm, tropical waters (Culik 2010). Its total distribution is uncertain because of past taxonomic confusion (Rice 1998). Common dolphins can frequently be seen with other marine mammal species and are often associated with schools of other cetaceans, particularly striped dolphins.

Harbor porpoises (*Phocoena phocoena*) are found in cool temperate and subpolar waters of the Northern Hemisphere (Jefferson et al 1993). In a study by Rosel et al. (1999), mitochondrial DNA analysis of porpoises from different Atlantic geographic origins showed that their movements are restricted. They are usually limited to coastal waters in the continental plate. In Portugal, this species is distributed along the coastal region but they mainly use the northern coast, in the area between Minho River and Nazaré. Genetics studies (Fontaine et al. 2007; Fontaine et al. 2010; Fontaine et al. 2014) that involved a significant number of Portuguese and Galician samples suggest that the porpoise population from the Iberian Peninsula and north-Africa is relatively isolated from the rest of the European populations. Iberian porpoises represent an isolated ecotype that could be proposed as a new subspecies in the future (*Phocoena phocoena meridionalis*) (Fontaine et al. 2014). They can be found in small groups of less than 8 individuals, although schools of 50 or more individuals have been described (Jefferson et al. 1993). They are not frequently seen in association with other species. Porpoises are listed in the Red Book of Portugal's Vertebrates as Vulnerable (Cabral et al. 2006).

Bottlenose dolphins are primarily a coastal species but may also be found in pelagic habitats throughout tropical to temperate waters, having a worldwide distribution (Reynolds III et al., 2000; Würsig and Pearson, 2015). Bottlenose dolphins live in a highly dynamic fission- fusion society, in which individuals associate in small groups with regular structure (composition and size) (Connor et al., 2000). Although there are many threats on local populations and several resident populations have shown declines over the last two decades (Augusto et al., 2012; Bejder et al., 2006; Guerra et al., 2014), the species is globally widespread and abundant, and a major population decline worldwide is not expected.

Marine mammals rely on healthy ecosystems for their survival and, being fully adapted to aquatic environments, they are uniquely suited to reflect ecosystem variability and degradation. Because marine mammals integrate and reflect ecological variation across large spatial and long temporal scales, they are prime sentinels of marine ecosystem change (Bossart 2011a; Nelms et al. 2021). In essence, the overall health of marine mammals

ultimately reflects the health of the ecosystems upon which they depend (Burek et al. 2008). Because they are top predators with considerable longevity and large body fat deposits, bioaccumulation makes them ideal candidates to monitor several types of pollution in marine systems. A bottom-up approach allows the monitoring of changes in lower trophic levels and a top-down approach sheds light onto the pressures of higher trophic levels, caused mainly by anthropogenic factors. Furthermore, they gather empathy from the general public, and changes to marine mammals' health can easily transmit a powerful message to people (Reddy et al. 2001; Moore 2008; Bossart 2011; Brito and Sousa 2011). Data on prevalence of diseases such as toxoplasmosis and brucellosis (Van Bressem et al. 2009) also highlights the importance of these species on the epidemiology of zoonotic diseases.

The number of stranded cetaceans per year along the Portuguese coast is relatively high allowing for the collection of data and representative samples (Vingada and Eira 2017), depending on funding availability. Since these data are not exactly equivalent to data obtained from live and *in situ* specimens, extrapolations to each respective population must be carefully considered. Nevertheless, the obtained data allows deepening the knowledge on cetaceans and opens a pathway to new conservation measures.

The RAMM is regionally operated by several organizations. Presently, most samples from the western coast are collected to the Marine Animal Tissue Bank (MATB) at ECOMARE –Universidade de Aveiro (UA), by technicians working for the University of Aveiro and the Portuguese Wildlife Society (SPVS). All technicians are licensed for capture, handling, tagging and sample collection in mainland Portugal under the Decree-Law n° 140/99 of 24th April and Decree-Law n° 49/2005 of 24th February, and Decree-Law n° 316/89 of 22nd September. Licenses are issued on a yearly basis by the ICNF. The Marine Animal Tissue Bank and the SPVS are registered in CITES with code PT009. Data and samples were collected according to standard protocols (Kuiken and Hartmann 1991) and each animal was identified using an alphanumeric code composed of the initials for the species, an attributed number, and the year of stranding.

Until recently, information concerning infectious diseases in cetaceans occurring in the Portuguese coast was scarce, although some data was available on the prevalence of skin conditions in common dolphins from the Sado estuary (Harzen and Brunnick 1997; Van Bressem et al. 2003). The FCT CETSENTI project (RECI/AAG-GLO/0470/2012) was a precursor in this research field in Portugal, building on a synergistic collaboration between the Faculty of Veterinary Medicine of the University of Lisbon (FMV-UL), UA, Universidade do Minho (UM) and SPVS. Among other aspects related with cetacean population health, the project included the first survey of CeMV and herpesvirus (HV) in several cetacean species inhabiting Portuguese waters. Also, since the immunological status of an individual plays an important role in the onset and establishment of disease, the first steps were taken with

respect to cetacean immunological response in relation with host health status. The occurrence of infectious disease is a complex process in the individual, and the determination of its significance at the population level is a daunting task (Gulland and Hall 2006). Host/pathogen/environment interactions that increase either the host's susceptibility or the pathogen's virulence or transmission rate may result in changes on the incidence of infectious disease. Data on baseline health parameters; identification and prevalence of pathogens; temporal and spatial patterns of disease and its relationship to environmental factors; effects of toxicants and host specificities, are all needed to understand the potential effects of climate change on marine mammal health (Burek et al. 2008).

2. Cetacean morbillivirus

2.1 Morbillivirus: characterization.

The genus *Morbillivirus* belongs to the Mononegavirales order, Paramyxoviridae family, which comprises 7 different genera. These are highly contagious viruses for their respective hosts and mediate similar consequences of pathogenesis in different species (fever, respiratory and gastrointestinal disease). Induction of a severe transient immunosuppression and the gain of life-long immunity in survivors are the most notable features of morbillivirus infection (Sato et al. 2012a). Affecting humans, measles is one of the most recognizable diseases caused by a morbillivirus, being a highly contagious infectious disease. It is a vaccine-preventable disease, but it has recently been associated to several outbreaks, even in countries where it was previously eradicated. In dogs, morbillivirus infection causes a disease called distemper, for which a vaccine is also available. Over the years, new morbilliviruses have been detected (see table 1) including a recently recognized feline morbillivirus (FeMV) associated with a persistent infection in cats causing tubule interstitial nephritis and chronic kidney disease (Woo et al. 2012; Choi et al. 2020).

In dogs and humans, chronic latent localized infections have been detected associated to defective forms of the virus and characterized by subacute sclerosing panencephalitis (SSPE) in humans (Measles virus) or old dog encephalitis (ODE) (Canine distemper virus), in dogs (Garg et al., 2019; Gutierrez et al 2010; Headley et al., 2009).

Table 1 – Known morbillivirus affecting different host species.

Species and Abbreviation	Host	Virus name
Measles Morbillivirus (MV)	Humans	Measles
Cetacean Morbillivirus (CeMV)	Cetaceans	Cetacean morbillivirus
Feline Morbillivirus (FeMV)	Cats	Feline morbillivirus
Phocine Morbillivirus (PMV)	Seals	Phocine morbillivirus
Rinderpest Morbillivirus (RPV)	Cattle and other Artiodactyla	Rinderpest
Small Ruminants Morbillivirus (PPRV)	Goats and Sheep	Peste des Petits Ruminants
Canine Morbillivirus (CDV)	Dogs	Canine Distemper Virus

Morbilliviruses are enveloped, non-segmented, negative-strand RNA viruses composed of six genes that encode eight proteins (as seen schematically in figure 2): the nucleoprotein (N), the polymerase-associated phosphoprotein (P) protein, the matrix (M), the surface glycoproteins F (fusion) and H (hemagglutinin), and the large (L) protein. The P gene encodes additional gene products, termed the V and C proteins, via an RNA editing process and an alternative translational initiation in a different reading frame, respectively. A viral RNA-dependent RNA polymerase composed of the L and P proteins associates with the nucleocapsid forming the ribonucleoprotein (RNP) complex, essential for the replication of morbilliviruses (Bellini et al 1985; Cattaneo et al 1989; Iwasaki et al., 2009).

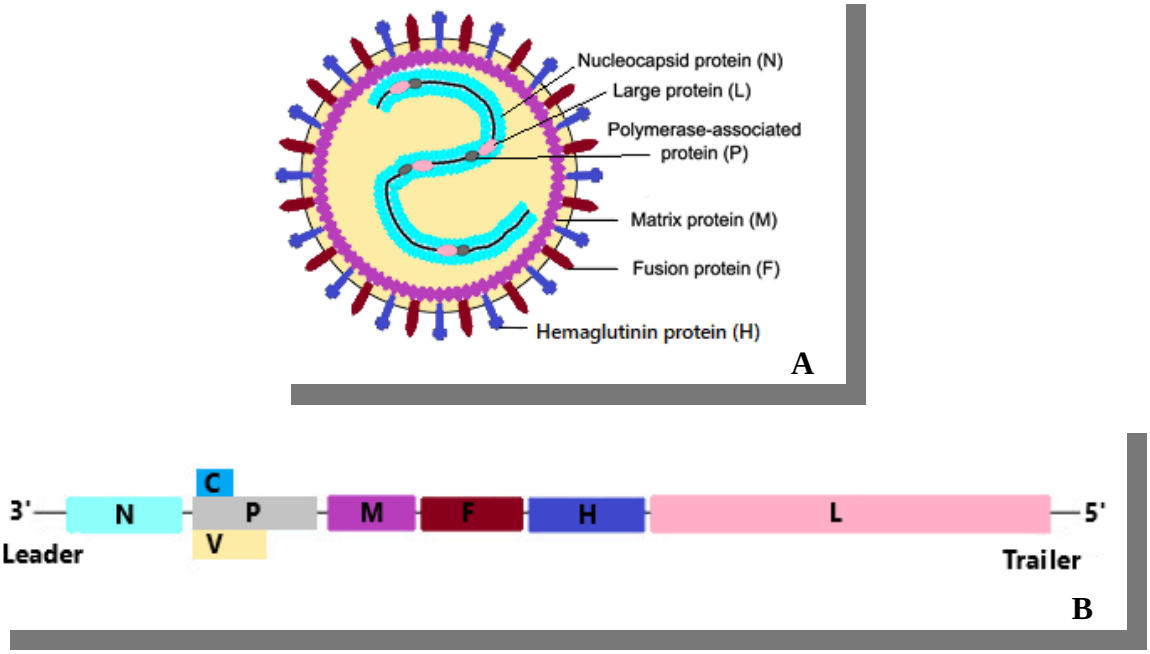


Figure 2 - Schematic representation of a morbillivirus virion (A) and its genome (B) - Adapted from Rima et al., 2019.

The H glycoprotein is responsible for virus attachment to the host cell membrane and for cellular entry. The F glycoprotein causes fusion with the host cell membrane and, together with the M protein, invokes cell-to-cell fusion (Banyard et al 2008; Wild et al 1991). H and F glycoproteins interact with cellular receptors that allow virus entry and determine host susceptibility, tissue tropism and viral pathogenesis (Delpeut et al 2014; Melia et al., 2014).

Investigations aimed at identifying the receptors for wild-type morbilliviruses started in 1993 with CD46, followed by the signaling lymphocytic activation molecule (SLAM) in 2000, and the epithelial cell receptor, nectin-4, in 2011 (Sato et al. 2012b). Most morbilliviruses, including MV, CDV, PDV, PPRV, and RPV, use the SLAM of their respective host species as a receptor (Adombi et al., 2011; Baron, 2005; Melia et al., 2014; Tatsuo et al 2001). The SLAM (or CD150) and the poliovirus like receptor 4 (PVLR4 or nectin 4) have both been recently identified as the major receptors for wild-type morbilliviruses in immune and polarized epithelial cells, respectively (Melia et al., 2014; Mühlebach et al., 2011; Noyce et al., 2011; Ohishi et al 2012; Shimizu et al., 2013b). CD147, a transmembrane glycoprotein that belongs to the immunoglobulin family and is present on a variety of cells including neuronal and endothelial cells, and the membrane bound form of heparin binding epithelial growth factor, have been suggested to function as entry receptors for MeV and PDV, respectively (Watanabe et al. 2010; Melia et al. 2014). SLAM is implicated in the regulation of T-cell activation by affecting T-cell antigen receptor signaling. In addition, SLAM regulates the functions of several other immune cell types, including natural killer and dendritic cells, having a broad involvement in the modulation of innate and acquired immune responses (Schwartzberg et al 2009; Veillette et al 2007; Veillette & Latour, 2003).

2.2 Epidemiology: hosts, history and current knowledge.

In marine mammals, the first reports of morbillivirus infection started in the late eighties and early nineties. The virus was reported in pinnipeds (Osterhaus et al. 1989; Visser et al. 1990; Bengtson et al. 1991), porpoises (Kennedy et al. 1988; Kennedy et al. 1991) and dolphins (Van Bressem et al. 1991). In 1993, Barrett et al. published a short communication stating that dolphin and porpoise morbillivirus were genetically distinct from phocine distemper virus. During the nineties, morbillivirus sequences found in cetaceans started to be denominated as a new virus called Cetacean morbillivirus, with two different strains: the dolphin morbillivirus and the porpoise morbillivirus. Approximately a decade after

the discovery of the first strains, a new strain was reported in pilot whales (Taubenberger et al. 2000) and designated Pilot Whale Morbillivirus (PWMV).

The SLAM receptor has been characterized in seven species of mysticetes and in 19 species of odontocetes (Cattaneo et al. 1989; Shimizu et al. 2013a). Among the nine cetacean families examined, variations were found between six amino acid residues, with charge alterations in four of them. Residue substitutions (G68, H90 and H130) that introduced charge alteration and possible change in viral affinity were observed in the SLAM of the *Delphinidae* family, whereas these residues were mostly conserved in the receptor of the other cetacean families. These changes could explain a possible higher dissemination and viral infectivity of CeMV in delphinid species, since disease die-offs were dominated by these species (Van Bressem et al., 2014). The only other odontocete cetacean that presented this H130Q variation was the porpoise, from the *Phocoenidae* family, a species that was greatly affected by morbillivirus infection in 1988–1990 (Kennedy et al., 1988).

As mentioned above, the first detection of the morbillivirus antigen in cetaceans occurred in porpoises stranded in the coasts of Ireland, England and the Netherlands in the late eighties, during a PDV-1 outbreak in seals. The virus was named Porpoise morbillivirus (Kennedy et al 1992; Kennedy et al., 1991, 1988; McCullough et al., 1991). Also during the eighties (1987-1988), a rise in mortality of bottlenose dolphins was detected on the west coast of the USA, specifically between New Jersey and Florida. A morbillivirus – Dolphin morbillivirus (DMV) – was detected (Lipscomb & Yvonne, 1994).

In 1990, DMV infection was detected in striped dolphins from the Mediterranean Sea (Van Bressem et al., 1991). Hundreds of dolphins were found dead along the Spanish coastline with signs of pneumonia and encephalitis (Domingo et al., 1992). Later, dead animals started to strand on the coasts of France, Italy and Morocco (Aguilar and Raga 1993). Since then, other outbreaks of the disease were reported in other countries. In 1993-1994 bottlenose dolphin deaths in the Gulf of Mexico were attributed to a DMV epidemic (Lipscomb et al., 1996); in 1994, 47 common dolphins died and stranded on the margins of the Black Sea and morbillivirus was detected by PCR (Birkun et al. 1999). Presently, this common dolphin population at the Black Sea is listed as endangered in the IUCN Red List (Birkun 2008).

Later on, serological studies showed a decrease in prevalence of CeMV seropositivity in striped dolphins from the Mediterranean. These results suggested that, after the 1990-92 outbreaks, the levels of protection against the disease were diminishing, rendering these animals susceptible to a new viral reintroduction (Van Bressem et al., 2001). In the same study, porpoises and common dolphins from the NE-Atlantic and the North Sea also revealed low antibody levels, suggesting that they were also at risk for CeMV infection.

In fact, after the early nineties outbreak in the Mediterranean, new outbreaks were identified. In 2007, a new rise in striped dolphins' mortality revealed a new epidemic of the disease. Detection of the virus by RT-PCR and N gene sequencing showed high similarity to the previously detected viral strain (Raga et al. 2008; Bellière et al. 2011). Between March and April 2011, the number of stranding events on the Valencian Community coast (Eastern Spain) increased: 37 dolphins were reported stranded over a 2-month period, which represented more than the annual average for that region, raising concerns of a new outbreak (Rubio-Guerri et al. 2013). Molecular biology analysis conducted in samples of these animals confirmed DMV infection.

A comparison between the striped dolphin outbreaks in 1990, 2007, and 2011 suggested a change in DMV epidemiology in the Western Mediterranean Sea. Mortality rates seem to be getting lower and lesions appear to be less severe, with mostly younger animals affected (Rubio-Guerri et al. 2013). It is possible that the DMV epidemiology in striped dolphins in the Western Mediterranean is changing from epizootic to enzootic infection (Domingo et al. 1995; Bellière et al. 2011; Rubio-Guerri et al. 2013).

Both the 90-92 and the 2007 DMV outbreaks started close to the Gibraltar Strait, suggesting that the viral introduction could have originated in the Atlantic populations. Serological evidence has been used to suggest that pilot whales may be reservoirs for DMV and that pilot whale populations are endemic for DMV whilst dolphins may represent a spill-over host. Several authors considered the pilot whale the most likely endemic source of morbilliviruses of cetaceans and a possible vector for its transmission to other species (Duignan et al. 1995; Van Bressem et al. 2001). Pilot whales have several characteristics that make them good candidates for being a CeMV endemic source and vector: 1) they move in large pods and have a cosmopolitan distribution and, 2) they are known to associate with different cetacean species. A high proportion of pilot whales sampled in the mid-1990s were seropositive for morbillivirus (Van Bressem et al. 2001).

The discovery of systemic morbillivirus infection in two adult striped dolphins stranded on the southwestern (Atlantic) coast of Spain, close to Gibraltar, in 2011 and 2012 further indicates that the Strait may play an important role in the epidemiology of CeMV (Soto, 2014). Furthermore, the detection of DMV strains in striped dolphins stranded in the Canary Islands identical to the Mediterranean strains (Van Bressem et al. 2014; Sierra et al. 2014) suggests that this population could also transmit the virus to the Mediterranean striped dolphins through occasional contacts through the Strait of Gibraltar.

Several studies found high prevalence of CeMV seropositive animals among populations of dusky dolphins (*Lagenorhynchus obscurus*), Fraser's dolphins (*Lagenodelphis hosei*) and melon-headed whales (*Peponocephala electra*). These species may also play a

role as reservoirs and vectors of the infection to susceptible species (Duignan et al. 1995; Van Bressem et al. 1998; Van Bressem et al. 2001; Duignan et al. 2006; Stone et al. 2012).

2.3 Morbillivirus pathogenesis: course of infection.

Pathology associated with CeMV is similar to what is commonly observed in other morbillivirus-infected animal and human hosts. Transmission occurs mostly after the inhalation of aerosolized virus shed by infected individuals. This form of transmission is also likely to occur among cetaceans and it should be favored by cetacean gregarious behavior and high density populations (Raga et al., 2008; Van Bressem, Van Waerebeek, & Raga, 1999).

Morbilliviruses are mostly lymphotropic and epitheliotropic and after initial replication in lymphoid tissue, the virus is disseminated by infected lymphocytes through the lymphatic system, spreading to epithelial cells (Appel, 1969; De Swart et al., 2007; Delpeut et al., 2014; Lemon et al., 2011; Ludlow et al 2015).

The course of infection can vary, and acute, sub-acute and chronic infections have been detected in cetaceans, as well as a chronic localized CeMV encephalitis (Van Bressem et al., 2014). An acute systemic disease has been observed, causing a severe multifocal or diffuse bronchopneumonia with interstitial edema. This infection is characterized by necrosis of type I pneumocytes and bronchiolar epithelial cells, and type II pneumocytes hyperplasia. Syncytial giant cells may form in alveolar and bronchiolar lumen and intracytoplasmic and intranuclear eosinophilic inclusion bodies are typically seen in acute morbillivirus infections. A severe generalized immunosuppression is a major feature in morbillivirus infection, and a non-suppurative encephalitis can be present in some animals. There are also cases of a subacute systemic form of the disease: animals did not succumb to the initial infection, but a profound immunosuppression facilitated secondary opportunistic infections by agents such as *Toxoplasma* sp. or *Aspergillus*. A focal non-suppurative demyelinating meningoencephalitis was also seen in these cases (Domingo et al., 1992; Stephens et al., 2014).

A chronic localized CeMV encephalitis, similar to the SSPE in humans and ODE in dogs, has also been identified in cetaceans with viral detection and lesions limited to the brain, without syncytial cells or inclusion bodies. This chronic localized infection represented the most common cause of stranding and death in mature striped dolphins in the years following a DMV epizootic (Soto et al., 2011). Several animals found dead in the period between 1991–1994 and 2008–2011 in the western Mediterranean and in 2009–2011 in the

Eastern Mediterranean presented this chronic encephalitis (Di Guardo et al., 2013; Domingo et al., 1995; Soto et al., 2011).

3. Cetacean Herpesvirus

3.1. Background and present data on epidemiology.

Herpesviruses fall within the taxonomic order Herpesvirales (Davison et al. 2009), composed of three families: Herpesviridae, affecting mammals, reptiles, and birds, Alloherpesviridae affecting fish and amphibians, and Malacoherpesviridae affecting bivalves. Within the Herpesviridae family, three subfamilies are recognized: *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae* (Davison et al. 2009; Pellett and Roizman 2013).

Viruses in the herpesviridae family are enveloped and vary in size (120-250 nm in diameter). They are spherical to pleomorphic with a nucleocapsid composed of 162 capsomers surrounded by an amorphous tegument with glycoprotein complexes embedded in the lipid envelope (Figure 3). The genome is linear double-stranded DNA with approximately 125-290 kb in size.

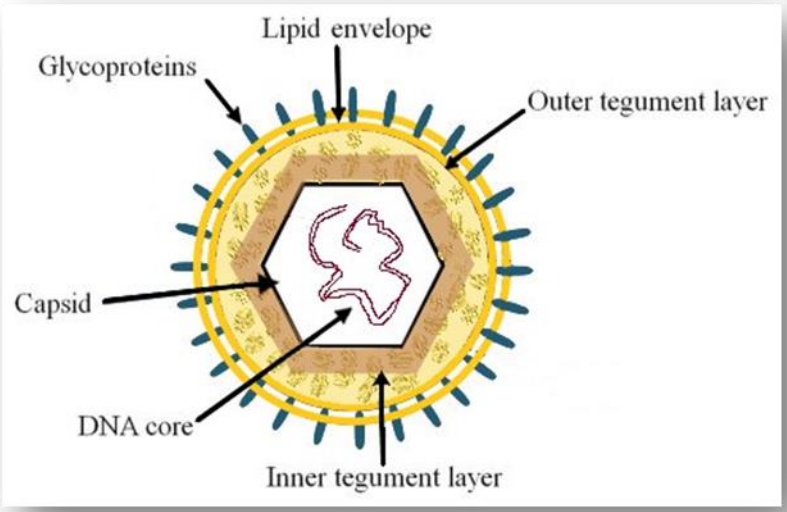


Figure 3 - Schematic representation of a herpesvirus virion, original image.

Replication occurs within the nucleus, with sequential transcription and translation of immediate early (α), early (β) and late (γ) genes. The earlier genes and their gene products regulate the transcription of later genes. Encapsidation also occurs in the nucleus and a budding process through the inner layer of the nuclear envelope forms the envelope. Infection results in characteristic eosinophilic intranuclear inclusion bodies. The number of open reading frames grossly underestimates the true genomic output of herpesviruses. In the case of cytomegalovirus (CMV), for example, alternative splicing and optional start codons give rise to more than 700 different viral proteins (Grinde 2013).

Herpesviruses are broadly considered species specific, because of long-term host-virus co-evolutionary processes. However, many herpesviruses may not be strictly host specific, and are capable of crossing species barriers and infecting different species, representing a zoonotic risk (Azab et al 2018). Although viruses with long-term evolutionary relationships with their host species tend to be relatively benign since killing their host would impede their replication and further progress, a recent zoonotic history is usually responsible for an increase in virulence.

The most characteristic property of herpesviruses is that they can establish latent infections in which viral gene expression is highly limited, with recrudescence and intermittent or continuous virus shedding, occurring in all herpesvirus infections. The latent genome is essentially silent, except for the production of a latency-related gene. This RNA transcript is not known to code for any protein; however, a small open reading frame (ORF-E) located within the latency-related gene appears to be expressed and inhibits apoptosis; the precise mechanisms responsible for the establishment, maintenance, and reactivation of latent infection are not fully characterized. Reactivation is usually associated with stress caused by intercurrent infections, shipping, cold, crowding, or by the administration of glucocorticoid drugs (MacLachlan and Dubovi 2011a).

In marine mammals the first known herpesvirus was the Phocid Herpesvirus 1 (PhHV-1), reported in 1985 in harbor seals in The Netherlands. Animals showed signs of acute pneumonia and viral hepatitis and an alphaherpesvirus was identified by electron microscopy and serum neutralization assays (Osterhaus et al 1985). A second herpesvirus was identified in harbor seal leukocytes' in 1994, and genetic analysis of the isolates in 1996 confirmed it to be a novel gammaherpesvirus – Phocid herpesvirus 2 (PhHV-2) (Lebich et al. 1994; Harder et al. 1996)

To this day, one alpaherpesvirus and four gammaherpesviruses have been identified in pinnipeds: Phocid Herpesvirus 1 and Phocid Herpesvirus 2, Hawaiian monk seal herpesvirus, otariine herpesvirus 1, and northern elephant seal herpesvirus (Osterhaus et al. 1985; Lebich et al. 1994; Harder et al. 1996; Lipscomb et al. 2000; Goldstein et al. 2006a; Goldstein et al. 2006b).

In cetaceans, herpesviruses were first identified by electron microscopy in skin biopsies from beluga whales in the late 80's (Barr et al 1989; Martineau et al., 1988). Later, also by electron microscopy, they were identified in harbor porpoises (*Phocoena phocoena*) (Kennedy et al., 1992) and in dusky dolphins (*Lagernorhynchus obscures*) (Van Bressem, Van Waerebeek et al 1994).

The first molecular diagnostic of herpesvirus in cetaceans took place in 2001 (Blanchard et al. 2001) in Atlantic bottlenose dolphins. Necropsy and histopathology of stranded animals revealed acute necrotizing lesions in several organs, with intranuclear eosinophilic inclusion bodies suggesting a disseminated herpesvirus infection. PCR targeting the DNA polymerase and terminase genes of HV was performed and phylogenetic analysis allowed the identification of two novel alphaherpesvirus.

Since then, HVs were identified in a myriad of odontocete cetaceans, such as: Blainville's beaked whale (*Mesoplodon densirostris*), dwarf sperm whale (*Kogia sima*) and Risso's dolphin (*Grampus griseus*) (Benson et al. 2006); Yangtze finless porpoise (*Neophocoena phocoenoides*) (Pei et al. 2012); Pacific white sided dolphin (*Lagenorhyncus obliquidens*) (Noguchi et al. 2013); Beluga (*Delphinapterus leucas*) (Bellehumeur et al. 2015); Striped dolphins (*Stenella coeruleoalba*) (Sierra et al. 2015; Bento et al. 2019); Common dolphins (*Delphinus delphis*) (Bento et al. 2019), and Harbor porpoises (*Phocoena phocoena*) (Elk et al. 2016; Bento et al. 2019). Herpesviruses have also been reported in mysticetes such as the fin whale (*Balaenoptera physalus*) and minke whale (*Balaenoptera acutorostrata*) (Melero et al 2015).

The advent of molecular biology techniques has allowed the identification of herpesvirus sequences in many different species, and the diversity of sequences found is remarkable. The possibility to amplify such a vast number of unknown sequences is vastly due to a set of primers designed for the DNA polymerase (DNApol) of herpesviruses, a rather conserved region, in a nested format conventional PCR which was first described by vanDevanter in 1996 (VanDevanter et al. 1996).

3.2 HV-associated pathology findings.

Herpesviruses have been found in cetaceans associated to a vast array of clinical signs. Some of the most frequently detected lesions associated with HV infection are cutaneous and mucosal lesions. In 2005, several skin and genital lesions were reported in stranded cetaceans that tested positive for herpesviruses in the nested conventional PCR

mentioned above (Benson et al. 2006). In Figure 4, skin lesions found in animals stranded in the Portuguese coastline can be seen.

Several other reports associated hyperplastic, proliferative skin, mucosal or genital lesions in cetaceans with herpesvirus infection. Lesions included: genital proliferative lesions in a striped dolphin (Sierra et al., 2015); proliferative dermatitis and genital lesions in Atlantic bottlenose dolphins (Van Elk et al 2009; Manire et al., 2006); genital proliferative plaques in Risso's dolphins, a dwarf sperm whale and in two Bainsville's beaked whales (Saliki et al. 2006; Benson et al. 2006) and proliferative dermatitis and genital lesions in beluga whales (Barr et al. 1989; Bellehumeur et al. 2015).

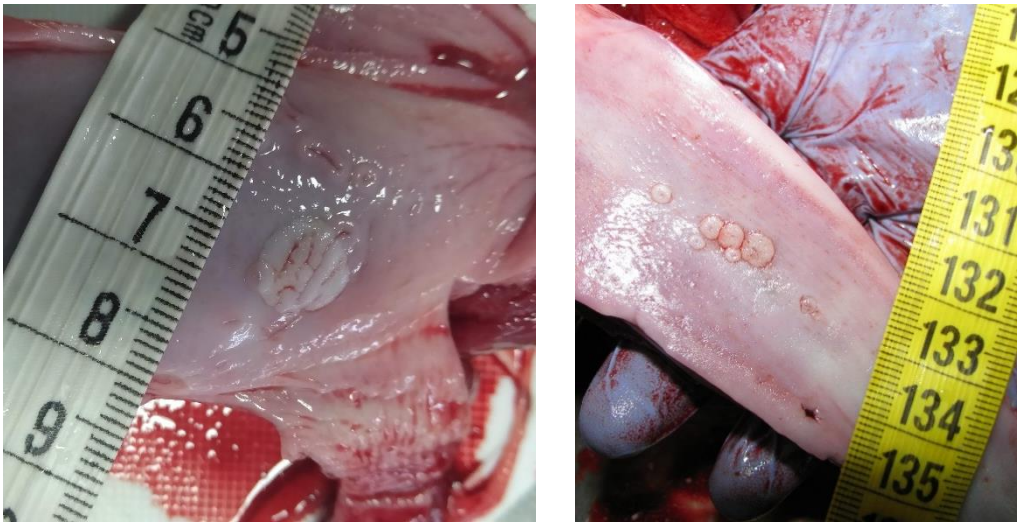


Figure 4 - Mucosal lesions detected during necropsies of cetaceans stranded along the Portuguese coastline: oral lesions in the tongue of two stranded common dolphins.

.

The oncogenic potential of these viruses has also been hypothesized. In other species, herpesvirus, more specifically gammaherpesvirus, can be associated with the proliferation of neoplasia. Viruses in this subfamily have a narrow host range, are lymphotropic, and become latent in lymphocytes; some are linked to oncogenic transformation of lymphocytes, notably *human herpesvirus 4* (Epstein–Barr virus), which is the cause of Burkitt's lymphoma and nasopharyngeal carcinoma in humans (MacLachlan and Dubovi 2011a). Another example of oncogenic herpesvirus is Human Herpesvirus 8 (HH8), causing Kaposi sarcoma, a low-grade vascular tumor.

Several oncogenic herpesviruses also affect other animal species. Marek's disease virus (MDV), more frequently referred to as Gallid herpesvirus 2 (GaHV-2), is the causative

agent of Marek's disease (MD) in chickens, a multifaceted disease most widely recognized by the induction of a malignant T-cell lymphoma. This major pathogen of poultry is the prototype species of the Mardivirus genus (Marek's disease-like viruses) within the Alphaherpesvirinae subfamily of the Herpesviridae family (Denesvre 2013). *Herpesvirus saimiri* (saimiriine herpesvirus 2) is the classical prototype of the gamma (2)-herpesviruses or rhadinoviruses, which also includes the Kaposi's sarcoma-associated herpesvirus. The T-lymphotropic *Herpesvirus saimiri* establishes specific replicative and persistent conditions in different primate host species. Virtually all squirrel monkeys (*Saimiri sciureus*) are persistently infected with this virus. In its natural host, the virus does not cause disease, whereas it induces fatal acute T-cell lymphoma in other monkey species after experimental infection (Fickenscher and Fleckenstein 2001).

In marine mammals, Otarine herpesvirus 1 (OtHV1) is strongly associated with California sea lion (CSL) (*Zalophus californianus*) urogenital carcinoma, the most common cancer documented in marine mammals. In addition to CSL, OtHV1 has also been found in association with carcinoma in South American fur seals (*Arctocephalus australis*), demonstrating the herpesvirus ability to infect related species (King et al. 2002; Buckles et al. 2006).

In cetaceans, a gammaherpesvirus has recently been detected in Indo-Pacific humpbacked dolphins (*Sousa chinensis*) with squamous cell carcinomas (Banlunara et al. 2019). This new herpesvirus has been tentatively named Sousa chinensis alphaherpesvirus. This report highlights an ongoing, aggressive epizootic of neoplastic disease in a captive population as well as photographic evidence suggestive of a similar process in several wild Indo Pacific humpbacked dolphins in Thai waters, strongly suggesting a contagious etiology. Although herpesvirus was detected in one of the animals tested, further studies are needed to determine if the viral infection is responsible for the neoplastic lesions (Banlunara et al. 2019).

4. Immunology

4.1 Cytokines as immunological assessment tools.

Cytokines are mainly produced by macrophages and lymphocytes and play an important role in antiviral immune responses by a wide range of mechanisms, including regulation of the expression of MHC molecules and co-stimulatory molecules and the direct activation or deactivation of immune cells. Cytokines are a large group of molecules and include the interferons (IFNs), interleukins (ILs), various colony-stimulating factors (CSFs),

the tumor necrosis factors (TNFs) and transforming growth factors (TGFs), thought to be particularly important in mediating inflammatory and cytotoxic reactions. This may lead to the activation of cellular antiviral responses involving NK cells and cytotoxic T cells, and antibody-mediated virus clearance (Ramshaw et al. 1997).

Playing a major role in the initiation and progression of immune responses, cytokines are good candidates to be used as tool for health assessment (Fonfara et al 2008). Cytokines may be divided into pro-inflammatory cytokines, such as interleukin IL1β, IL6, IL8, IL12, and TNFα, and anti-inflammatory cytokines, like IL-4 and IL10 (Fonfara et al. 2008). In the early stages of immune response, pro-inflammatory cytokines are produced mainly by macrophages and monocytes and may be used to detect subclinical infections (Funke et al 2003; King et al., 1996). Cytokine expression in this early phase is dominated by the expression of TNF-α, IFN-γ and IL-1 by activated macrophages and lymphocytes, inducing the production of other pro-inflammatory cytokines and inflammatory mediators (acute phase response) (Roth and De Souza 2001).

Cytokines play a major role in shaping immune response as type-1 cytokines elicit predominantly cell-mediated immunity and type-2 cytokines a predominantly humoral immunity (Patel et al. 2012). Cytokines directing Th1 and Th2 responses, and produced by the respective subsets, have been shown to cross-regulate each other's development and function. T lymphocytes are a major source of cytokines and they bear antigen specific receptors on their surface to allow recognition of foreign pathogens. There are two main subsets of T lymphocytes, depending on their surface molecules, known as CD4 or CD8. T lymphocytes expressing CD4, known as helper T cells (Th cells), are the most prolific cytokine producers. This subset can be further subdivided into Th1 and Th2, and the cytokines they produce are known as Th1 type cytokines and Th2 type cytokines (Berger 2000). Th1 cells secrete pro-inflammatory cytokines, with a special emphasis on IFNγ, IL2 and TNFα, stimulating cell-mediated immunity. The Th2 type cytokines include interleukins 4, 5, 13, and also IL10, which leads to an anti-inflammatory response, inhibiting cell mediated immunity and promoting humoral immune responses (Berger 2000; Fonfara et al. 2008).

Several authors have investigated cytokine expression for the assessment of immune function in marine mammals; however, scarce information is available on their association with the health status in marine species. In a study on harbor porpoises, increased concentrations of IL10 mRNA correlated with an impaired health status. It was hypothesized that elevated IL10 levels could reflect a continuous stimulation of the immune system in chronically diseased harbor porpoises (Beineke et al 2007). These results indicate that IL10 levels can be regarded as a potential marker for chronic infectious diseases, as previously described for other species (Rigopoulou et al 2005; Song et al 1999).

Both inflammatory and anti-inflammatory reactions are normal components of the same immune response, which coordinately fight infections while preventing immune pathology. In the late phase, anti-inflammatory cytokines, such as TGF-b, IL-4 and IL-10 produced by lymphoid cells terminate the immune response. IL-10 is an important suppressive cytokine, produced by a large number of immune cells, and a key player in anti-inflammatory immune responses (O'Garra et al, 2004).

Cytokines have been used to determine prognosis and even to help diagnose several diseases through the evaluation of their expression levels and different profiles. In humans, quantification of IFNγ can be used as a diagnostic tool for tuberculosis (Santín Cerezales and Benítez 2011) and cytokine profiling has been studied as a method of distinguishing between latent and active infection (Won et al. 2017). In cats, cytokines have been studied as a diagnostic tool for *Mycobacterium* infection (O'Halloran et al. 2018). Cytokine markers have also been studied, either as a prognostic or as diagnostic tool, in other viral diseases such as varicella (Hao et al. 2015), bovine HV 5 (Cardoso et al. 2016), covid-19 (Han et al. 2020), among others. Cytokine expression profile has particularly been important in the study of sepsis establishment and evolution (Chousterman et al 2017; Faix, 2013; Grondman et al 2020).

Viral infections pose a major challenge to host survival, and the capacity of the virus to replicate and persist in the host is challenged by the host's antiviral defense mechanisms. Antiviral defense mechanisms are numerous and range from non-specific defenses to sophisticated mechanisms specifically induced in response to viral antigens. The complexity of such mechanisms, with distinct yet overlapping roles, is proof of their importance in host survival as an outcome of a virus infection (Ramshaw et al. 1997).

Experiments using mice indicate that IFNs are essential for innate immunity against viral infections. IFN-α, IFNγ and IFN-β are responsible for the response to viral and bacterial pathogens and parasites. Interferons perform multiple functions during viral infections by inhibiting viral replication and spread. Furthermore, they modulate immunity, both innate mechanisms and adaptive responses, shifting it towards cellular cytotoxicity, and exert an anti-proliferative influence on some types of cells (De Veer et al. 2001). Mice with a targeted disruption of the type I or II IFN receptor genes were extremely susceptible to viral infections, showing enhanced viral replication in many tissues (Müller et al. 1994).

Morbillivirus infection has similar traits across species: they usually cause a respiratory infection with pneumonia and encephalitis, and a marked immunosuppression in the host. The effects of morbillivirus in the immune system have been extensively studied in humans, with Measles being an important disease due to its severity, mainly affecting populations in developing countries with restricted access to vaccines and overall healthcare. These populations also suffer with malnutrition and other infectious diseases, which

aggravate even more an already severe infection. MV infection is accompanied by severe suppression of both innate and adaptive immune responses that may last for months and is caused via multiple mechanisms (de Vries et al. 2012; de Vries and de Swart 2014).

Inflammation markers could be helpful to monitor the health status of marine mammals, both in captivity and in free-ranging animals, especially considering that inflammatory processes and the impairment of immune function can be difficult to detect in these species (King et al. 1996, Zeneto-Savin et al. 1997, Funke et al. 2003).

5. Objectives

This work has been developed in the last five years by a team of multidisciplinary contributors that include biologists, veterinarians and veterinary nurses. This collaboration allowed collecting a valuable body of samples and knowledge, which was integrated in order to shed light onto the life and death of free-roaming cetacean species off the coast of Portugal. The overall aim of the present study was to characterize morbillivirus and herpesvirus infections and their repercussions in the health of the sampled cetacean populations. The specific objectives of the present study were:

- To clarify the prevalence of CeMV in cetacean populations from the eastern Atlantic, and to investigate the relationship between the dolphin morbillivirus strains circulating in the sampled eastern Atlantic areas and elsewhere in the world, with particular emphasis to the Mediterranean.
- To identify novel or previously described herpesviruses in samples from cetaceans stranded on the Portuguese coastline and to determine relations between these new herpesvirus sequences and representatives of the known Herpesviridae subfamilies and genera, available at GenBank ® as cetacean herpesvirus sequences.
- To evaluate the relative expression of different cytokine genes (IL1β, IL6, TNFα, IL12, IL4,IL10, and IFN-γ) in a group of healthy common dolphins, in order to establish a baseline profile to be used in the future as a health assessment tool, and to compare cytokine profiles of CeMV-infected individuals with a control group to infer changes in their immune system caused by this viral infection.

In general, the results obtained in this work will contribute to a better understanding of the epidemiology of these viruses in cetacean populations living in the Eastern Atlantic, as well as their effect on their population health.

CHAPTER II

New insight into dolphins morbillivirus phylogeny and epidemiology in the northeast Atlantic: opportunistic study in cetaceans stranded along the Portuguese and Galician Coasts

---

Bento MC, Eira CI, Vingada JV, Marçalo AL, Ferreira MC, Fernandez AL, Tavares LM, Duarte AI. 2016. BMC Veterinary Research. 2016 Aug 26;12(1):176. doi: 10.1186/s12917-016-0795-4. PMID: 27566667; PMCID: PMC5002201

Abstract

Screening Atlantic cetacean populations for Cetacean Morbillivirus (CeMV) is essential to understand the epidemiology of the disease. In Europe, Portugal and Spain have the highest cetacean stranding rates, mostly due to the vast extension of coastline. Morbillivirus infection has been associated with high morbidity and mortality in cetaceans, especially in outbreaks reported in the Mediterranean Sea. However, scarce information is available regarding this disease in cetaceans from the North-East Atlantic populations. The presence of CeMV genomic RNA was investigated by reverse transcription-quantitative PCR in samples from 279 specimens stranded along the Portuguese and Galician coastlines collected between 2004 and 2015.

A total of sixteen animals (*n* = 16/279, 5.7 %) were positive. The highest prevalence of DMV was registered in striped dolphins (*Stenella coeruleoalba*) (*n* = 14/69; 20.3 %), slightly higher in those collected in Galicia (*n* = 8/33; 24.2 %) than in Portugal (*n* = 6/36; 16.7 %).

Phylogenetic analysis revealed that, despite the low genetic distances between samples, the high posterior probability (PP) values obtained strongly support the separation of the Portuguese and Galician sequences in an independent branch, separately from samples from the Mediterranean and the Canary Islands. Furthermore, evidence suggests an endemic rather than an epidemic situation in the striped dolphin populations from Portugal and Galicia, since no outbreaks have been detected and positive samples have been detected annually since 2007, indicating that this virus is actively circulating in these populations and reaching prevalence values as high as 24 % among the Galician samples tested.

1. Introduction

Morbillivirus infection affects mainly the upper respiratory tract, central nervous system and the immune system (Beineke et al 2010; Van Bressem et al., 2014) and has been identified as a cause of death and stranding in marine mammals (Domingo et al. 1992). In odontecetes, infection has been associated with high mortality rates occurring during disease outbreaks in different parts of the world (Van Bressem et al., 1999). The mortality rate in striped dolphins from the Mediterranean Sea in the beginning of the nineties was the highest recorded so far (Aguilar & Raga, 1993; Forcada et al 1994; Van Bressem et al., 2014). Further studies are needed to deepen the knowledge about this disease. An

integrated approach taking into consideration epidemiological and environmental parameters should provide a better picture of the ecology and evolution of Cetacean Morbillivirus (CeMV) in free-ranging cetaceans (Van Bressem et al., 2014).

CeMV includes three well characterized viral strains (Van Bressem et al. 2009): porpoise morbillivirus (PMV), dolphin morbillivirus (DMV) and pilot whale morbillivirus (PWMV); three novel cetacean morbillivirus strains were recently reported (West et al. 2012; Groch et al. 2014; Stephens et al. 2014), adding to the genetic diversity of these viruses.

Morbilliviruses affecting cetaceans have been described in the last decades (Van Bressem et al. 2014) after the initial detection of viral antigens in these species in the late eighties. The first evidence of morbillivirus infection in cetaceans occurred in 1988 during a PMV outbreak, when the viral antigen was detected in harbour porpoises (*Phocoena phocoena*) stranded in Ireland (Kennedy et al., 1988). In the early nineties, dolphin morbillivirus (DMV) was isolated from striped dolphins from the Mediterranean (Domingo et al., 1990; Van Bressem et al., 1991); in 2000 PWMV was first described in a long-finned pilot whale (*Globicephala melas*) from the US coast (Taubenberger et al. 2000) and later, in 2011, from a short-finned pilot whale (*Globicephala macrorynchus*) in the Canary Islands (Bellière et al., 2011).

Due to the virus pathogenic impact on cetacean populations, further information about morbillivirus infection in cetaceans worldwide is relevant to understand its epidemiology in these animals. Studying infectious diseases in these species is important, especially considering that additional non-infectious aggressions, mainly due to human activities, render these populations even more susceptible to disease. An annual average of 200 stranded cetaceans were registered between 2010 and 2012, considering the Algarve and the Northern region of the Portuguese continental coast (Vingada et al. 2012) and fisheries bycatch was identified as the most significant cause of death. To this date, no molecular data was published on morbillivirus infection in animals stranded in Portugal or northern Spain. In 2014, dolphin morbillivirus infection was reported in a retrospective study affecting striped dolphins and a common dolphin from the Canary Islands (Sierra et al., 2014), causing non-suppurative meningoencephalitis. Also, a fatal systemic morbillivirus infection was detected in a bottlenose dolphin stranded in 2005 in the Canary Islands (Sierra et al., 2014). It was suggested that DMV was not endemic in harbour porpoises and common dolphins (*Delphinus delphis*) from the NE Atlantic (British Isles) in the period 1996–1999 (Van Bressem et al., 2001), as low antibodies titers were detected in animals from Spain and the North Sea.

DMV infection apparently did not persist as an endemic infection in Mediterranean striped dolphins after the 1990–92 epidemic (Van Bressem et al., 2001). Both epidemics in the Mediterranean Sea (1990–92 and 2006–07) started near the Gibraltar Strait (Raga et al.,

2008) and it has been suggested that DMV-infected cetaceans may have entered the Strait of Gibraltar and infected striped dolphins, the most common cetacean at the time (Di Sciara et al., 1993; Van Bressem et al., 2009). Pilot whales had been already proposed as reservoirs in 1995 (Duignan et al., 1995). Later, in 2006 several long-finned pilot whales were found stranded along the coast of the Alboran Sea, and morbillivirus infection was detected (Fernández et al. 2008). In this epidemic, deaths were first detected close to the Gibraltar Strait and spread further into the Mediterranean Sea. Recently described sequences found in striped dolphins from the Canary Islands show high identity with sequences from the Mediterranean outbreaks, indicating the possible circulation of viruses between the Atlantic and the Mediterranean (Sierra et al. 2014). The role of other cetacean species as reservoirs needs to be further assessed.

The objective of the present study was to clarify not only the prevalence of DMV in cetacean populations from the eastern Atlantic, but also to investigate the relationship between the dolphin morbillivirus circulating in the eastern Atlantic and elsewhere in the world, especially in the Mediterranean.

2. Methods

2.1 Sample collection

Stranded cetaceans were collected by the Sociedade Portuguesa de Vida Selvagem (SPVS) in Northern Portugal and the Algarve within the Marine Animal Stranding Network, managed by the Instituto para a Conservação da Natureza e Florestas (ICNF) and in Galicia by the Coordinadora para o Estudo dos Mamiferos Mariños (CEMMA). Permission was issued by the National Authority (ICNF) to SPVS technicians to collect wildlife samples within the national territory according to laws n.140/99, n.49/2005, n.156-A/2013, and n.316/89. Also, SPVS is a registered CITES scientific research institution (code PT009). CEMMA holds a permit from the Conselleria de Medio Ambiente, Territorio e Infraestruturas de Xunta de Galicia (Spain) to collect and maintain cetacean samples according to law 42/2007 and law 9/2001.

The animals were assigned a decomposition code (1 to 5) according to already established protocols (Geraci and Lounsbury 1993). Animals with a score ranging from to 1 to 3 (fresh to moderate decomposition) were surveyed in the present study. During necropsy, tissue samples were collected from 279 cetaceans: brain, lung, pulmonary lymph node, mesenteric lymph node, spleen, kidney and liver, whenever possible. For animals from Galicia the only available sample was the lung. Samples collected in Portugal were stored in

vials with RNAlater® at −20 °C and samples collected in Galicia were frozen at −20 °C. All samples were kept in the marine animals' tissue banks (MATBs) of SPVS and CEMMA. Samples from different species were collected between 2004 and 2015: common dolphins (DD), striped dolphins (SC), bottlenose dolphins (*Tursiops truncatus*; TT), long-finned pilot whales (*Globicephala melas*; GM), Pigmy Sperm Whale (*Kogia breviceps*; KB), True's Beaked Whale (*Mesoplodon mirus*; MMi) and Fin whale (*Balaenoptera physalus*; BP) (Table 2). Samples were identified with a code composed by the species identification (e.g., DD, SC, TT), a number attributed to each stranding, and the year of stranding. From cetaceans stranded in the Portuguese coastline 91 animals from 2011, 56 from 2012, 33 from 2013, 49 from 2014 and 7 from 2015 were tested. From Galicia, a total of 33 lung samples from striped dolphins were tested. Available tissue samples from 10 animals stranded in Portugal from previous years were also included in this study (6 striped dolphins and 4 pilot whales from 2004 to 2009).

Table 2 – Number of stranded cetaceans tested for DMV per year.

		2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	Total
Portugal	Common dolphin (DD)	-	-	-	-	-	-	-	84	45	29	29	6	193
	Striped dolphin (SC)	1	-	1	4	-	-	-	5	6	3	16	-	36
	Pilot whale (GM)	-	-	-	-	2	2	-	-	1	-	-	-	5
	Bottlenose dolphin (TT)	-	-	-	-	-	-	-	1	2	1	3	-	7
	True's beaked whale (MMi)	-	-	-	-	-	-	-	1	-	-	-	-	1
	Pigmy Sperm Whale (KB)	-	-	-	-	-	-	-	-	1	-	1	-	2
	Fin whale (BP)	-	-	-	-	-	-	-	-	1	-	-	1	2
Galicia	Striped dolphin (SC)	2	1	1	2	3	6	4	2	3	5	4	-	33

2.2 Total RNA extraction

Total RNA was extracted from a pool of tissue homogenates using RNeasy mini kit (Qiagen, GmbH, Germany), according to the manufacturer's instructions. The pool included, whenever possible: lung, brain, pulmonary lymph node and mesenteric lymph node. Total RNA quantification and purity was determined using a Nanodrop 2000C spectrophotometer (ThermoScientific, USA) and stored at −80 °C until used.

2.3 Detection of dolphin morbillivirus genomic RNA by reverse transcription-quantitative PCR (RT-qPCR)

The detection of viral RNA for the DMV strain of CeMV was performed by RT-qPCR in a StepOnePlus thermocycler (Applied Biosystems), using primers (Stabvida genomics lab, Portugal) and probe (Eurogentec), targeting the N gene of DMV, as previously described (Grant et al. 2009) (Table 3). A previously detected positive sample for DMV was used as a positive control of the PCR reaction. Negative reaction controls were always included.

Table 3 - Primers and probe set used in RT-qPCR assays.

	5′ Fluorophore	3′ Quencher	Sequences (5′–3′)	Amplicon size	Annealing (°C)	
DMV-N-FP	–	–	TGCCAGTACTCCAGGGAACATCCTTC	173	60	Grant et al 2009
DMV-N-RP	–	–	TTGGGTCGTCAGTGTTGTCGGACCGTT	173	60	
DMV-N-probe	Cy3	BHQ1	A + CA + CCAAA + AGGGA + CA	–	60	

One step RT-qPCR assays were performed using 100 ng of the template RNA, in a total reaction volume of 20 µL containing: 10 µL of 1-step qPCR-ROX Mix (2×); 1 µL of RT enhancer; 0,2 µL of Verso Enzyme Mix (Verso 1-Step qRT-PCR ROX kit, ThermoScientific®); 0.4 µM of each primer and 0.25 µM of probe. For positive samples, total RNA was extracted individually for each of the available organs and the infection was evaluated individually in the different organs. The amplified DMV fragment was cloned into a plasmid vector (Pgem Teasy – Promega) and serial tenfold dilutions of the recombinant plasmid DNA were used to construct the standard curve (Fig. 5). The results showed a high correlation ($R^2 = 0.997$) with a calculated efficiency of 81 %. The primers and probe could detect viral RNA copies down to 10^2, and the limit of detection was 224 copies.

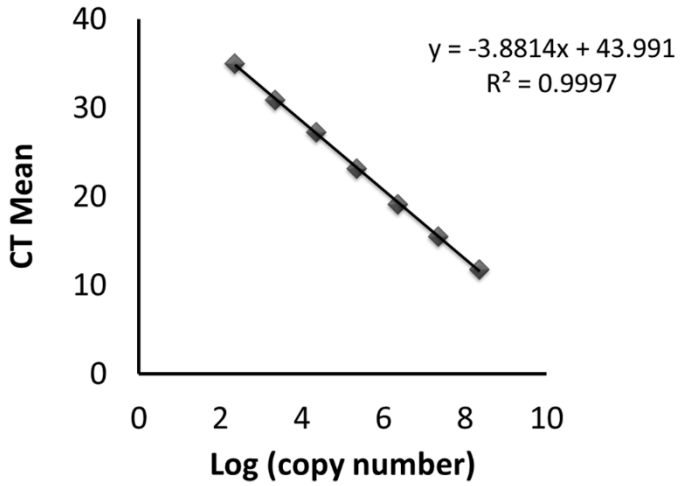


Figure 5 – Standard curve and equation for the determination of the efficiency of the RT-qPCR for the molecular detection of DMV.

2.4 Conventional PCR for amplification of DMV genes.

Additional sequences were amplified from the positive samples by conventional reverse transcription-PCR (RT-PCR) using previously described primers (Table 4) purchased from Stabvida genomics lab (Portugal). Primers were used in different combinations, targeting different genomic regions (Fig. 6).

Table 4 - Primers used in conventional PCR assays.

Primer	Target gene	Sequence (5'-3') (sense)	Tm	Genome position	Reference
CeMV-He1	H	CRTTGATACTYGTGGGTGTG (+)	59	7194–7213	Bellière et al 2011
CeMV-He2	H	TGTTAACTTCTGGGGCATCC (-)	59	7407–7426	
DMVFu-F	F	GGCACCATAATTAGCCAGGA (+)	51	6483–6502	
DMVFu-R	F	GCCCAGATTTGTGCCTACAT (-)	51	6655–6674	
DMV-C	P	ATGTTTATGATCACAGCGGT (+)	51	2132–2151	Grant et al 2009
DMV-P2	P	ATTGGGTTGCACCACTTGTC (-)	51	2541–2560	
NgeneF	N	CCHAGRATYGCTGAAATGATHTGTGA (+)	48	849–874	Taubenberger et al 20000
NgeneR	N	AACTTGTTCTGRATWGAGTTYTC (-)	48	1056–1078	

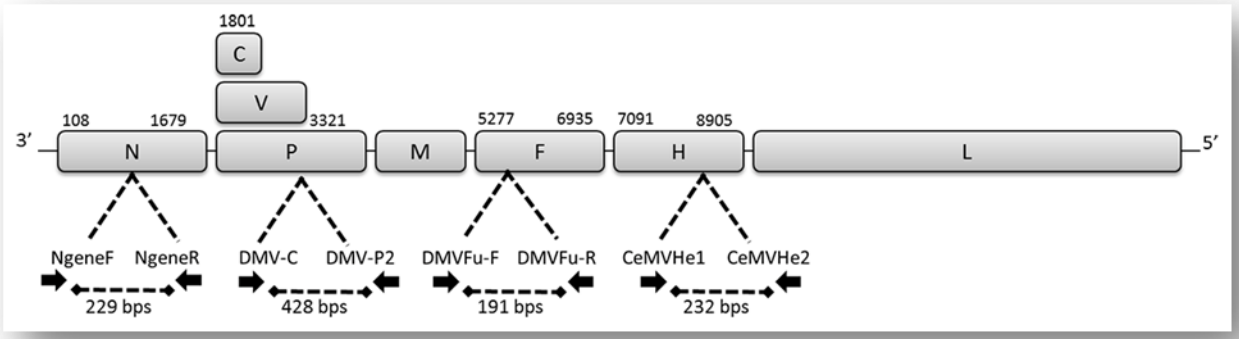


Figure 6 - Schematic representation of the primers used to amplifiy different genomic regions by conventional RT-PCR.

The obtained amplicons were used to perform a phylogenetic analysis of the DMV sequences, along with sequences retrieved from NCBI for the same genes. L and M genes were not targeted in the conventional RT-PCR since very few sequences were available at the NCBI database.

The amplicons were directly sequenced by Sanger sequencing at Stabvida, Portugal and the specificity of the nucleotide sequences was compared by Blast analysis http://blast.ncbi.nlm.nih.gov/Blast.cgi with CeMV sequences available in the GenBank.

2.5 Phylogenetic analysis

The nucleotide sequences of the Portuguese and Galician sequence datasets available in the GenBank (National Center for Biotechnology Information) repository ,with the following accession numbers KP835987; KP835991; KP835995; KP835999; KP836003; KP835986; KP835990; KP835994; KP835997; KP836002; KP836006; KP835985; KP835989; KP835993; KP835996; KP836001; KP836005; KP835984; KP835988; KP835992; KP835998; KP836000; KP836004; KP835983; KT878649; KT878650; KT878651; KT878652; KT878653; KT878654; KT878655; KT878656; KT878657; KT878658; KT878659; KT878660; KT878661, were compared with the available CeMV sequences and outgroup taxa (Canine Distemper Virus [CDV], Phocine Distemper Virus [PDV] and Measles Virus [MV]), retrieved from GenBank (table 5), according to their primary structure similarity using the multiple alignment ClustalW program (Thompson et al. 1994).

Table 5 – Accession number for GenBank sequences used to the phylogenetic analysis and corresponding description.

Complete genomes and common sequences	NC_0014981	Measles Virus	AY649446	Canine Distemper Virus
	KC802221	Phocine Distemper Virus	AJ608288	Dolphin Morbillivirus complete genome
	HQ829973	Striped dolphin 2007 SP (Med)	HQ829972	Long-finned pilot whale 2007 SP (Med)
Gene N	X84739	Porpoise 1988 IRL	AF200818	Long-finned pilot whale 1999 USA
	FJ842380	Short-finned pilot whale 1996 SP (Can Isl)		
Gene P	KF695110	Bottlenose dolphin 2005 SP (Can Isl)	JX195718	Longman's beaked whale 2010 USA
	EU039963	Long-finned pilot whale 2007 SP	KF650727	Porpoise 1990 NL
	EF451565	White-beaked dolphin 2007 GM	AF200817	Long-finned pilot whale 1999 USA
	AF333347	Pigmy sperm whale 2001 TW	KJ139451	Striped dolphin 2002 SP (Can Isl)
	KJ139452	Striped dolphin 2007 SP (Can Isl)	JN210891	Striped dolphin 2011 SP (Med)
	KF711855 KC572861 KR337460 KC888945	Guiana dolphin 2010 BR Striped dolphin 2012 SP (Med) Fin whale 2013 IT White-beaked dolphin 2011 NL	KJ139454 KJ139453 KR704575	Striped dolphin 2011 SP (Can Isl) Striped dolphin 2009 SP (Can Isl) Longman's beaked whale 2013 NC
Gene F	AJ224704	Striped dolphin 90's SP	Z30086	DMV 1994
	FJ842382	Short-finned pilot whale 1996 SP		
Gene H	FJ648457	Porpoise MV 1988 IRL	AJ224705	Striped dolphin 90's SP
	Z36978	DMV 1994	FJ842382	Short-finned pilot whale 1996 SP (Can Isl)

SP=Spain; (MED)=Mediterranean; (Can Isl)=Canary Islands; IRL=Ireland; USA=United States of America; NL=Netherlands; GM=Germany; TW=Taiwan; BR=Brazil, IT=Italy, NC=New Caledonia.

Six sets of alignments were considered for the phylogenetic analysis: nucleotide sequence alignments for genes N, P, F and H, composed by sequences of 218, 342, 449 and 316 base pairs, respectively; concatenated sequence of amino acids (540 aa) and nucleotides (1446 bps). Due to heterogeneity of the available DMV sequences it was not feasible to maintain the same set of DMV sequences in the alignment for each gene. In the

concatenated alignment only the sequences with all partial genomic regions were included. The multiple sequence alignments were manually corrected with Jalview, Version 2.0.1 (Waterhouse et al 2009) removing long internal gaps and unmatched ends to maximize genetic similarities and phylogenetic trees were inferred by Bayesian methods (MrBayes v.3.2.1) (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003).

For the Bayesian analysis a Markov chain Monte Carlo (mcmc) simulation technique was carried out to approximate the PP of trees (Ronquist and Huelsenbeck 2003). The evolutionary GTR (nucleotides) and LG (amino acids) models were selected with gamma-distributed rate variation across sites and a proportion of invariable sites (rates = invgamma). The analysis was initiated using a random tree from the dataset with four chains running simultaneously for 20×10^6 generations, sampling every 100 generations. The first 25 % trees were discarded, and a majority rule consensus tree was generated from the remaining trees. The graphical representation and edition of the phylogenetic tree were performed with FigTree v1.3.1. Only support values equal or greater than 0.70 of PP are shown in the trees.

2.6 Statistical analysis

Chi-square test of association was performed to assess if the difference in prevalence was statistically significant between different species (DD and SC) and between animals from different origins (Portugal and Galicia). For this analysis an online website for statistical computation was used url: http://vassarstats.net/. A confidence interval (CI) of 95 % (for a *p* value ≤0.05) was considered for all the statistical analysis.

3. Results

A total of 16 DMV positive cetaceans were identified by RT-qPCR, representing a prevalence of 5.7 % (IC95 %: 3.42;9.32). With respect to the Portuguese coastline, 8 positive animals were detected, including 6 striped dolphins (SC) and 2 common dolphins (DD) [SC/15/2007, SC/257/2011, SC/221/2012, DD/302/2012, SC/11/2013, DD/191/2013, SC/193/2014 and SC/290/2014]. In Galicia, 8 positive striped dolphins were detected [SC/21/2007, SC/24/2008, SC/31/2009, SC/42/2010, SC/49/2011, SC/51/2012, SC/53/2012 and SC/55/2012]. Among all cetacean species, striped dolphins ($n = 69$) revealed a significantly higher DMV prevalence reaching 20.3 % (IC95 %: 11.92; 32.02), whereas common dolphins ($n = 139$) recorded a prevalence of 1.0 % (IC95 %: 0.18; 4.09) (*P* value *0.00*). Positive striped dolphins were detected every year (from 2007 to 2014) while positive common dolphins were only detected in 2012 and 2013. The DMV prevalence in

striped dolphins stranded in Galicia was 24.2 % (IC95 %: 11.74; 42.63) whereas in Portugal the DMV prevalence was 16.7 % (IC95 %: 6.97; 33.47). From the positive animals stranded along the Portuguese coastline, each organ included in the tissue pool was tested individually for viral RNA. Two animals tested positive in all available organs; four were positive for viral RNA only in the brain and one animal tested positive in the lung, and in the pulmonary and mesenteric lymph node (Table 6). Lung was the only available sample to test in samples from Galicia.

Table 6 – Mapping of DMV infection in the available organs in Portuguese samples.

	Lung	Brain	Pulmonary LN	Kidney	Spleen	Liver	Mesenteric LN
SC/257/2011		X					
SC/221/2012	X	X	X	X	X	X	X
DD/302/2012	X		X				X
SC/11/2013		X					
DD/191/2013	X	X	X	X	X	X	X
SC/290/2014		X					
SC/193/2014		X					
SC/15/2007*	X						

Tested organs are shown in grey and positive organs are marked with an (X).

For samples SC/15/2007, SC/257/2011, SC/53/2012, SC/55/2012, DD/302/2012, SC/290/2014, SC/11/2013, SC/31/2009, SC/51/2012, SC/21/2007, SC/221/2012 and DD/191/2013 longer genomic regions were amplified by one step RT-conventional PCR with the primers described previously (Table 4). For samples SC/24/2008, SC/42/2010, SC/49/2011 and SC/193/2014 no fragments were amplified by conventional RT-PCR.

For the nucleotide sequences of each genomic region, phylogenetic trees were inferred by Bayesian methods. All trees exhibited a similar sequence topology, supported by robust PP values, regardless of the total number of sequences in each tree.

In the tree of the concatenated nucleotide sequences (Fig. 7) the CeMV sequences were distributed in three main branches supported by high PP values. Portuguese and Galician samples from 2011, 2012 and 2013 were included in one branch; sequences from the Mediterranean from 2007, early nineties (AJ608288) and the Portuguese sequence SC/15/2007 in another branch. The only PWMV included in this tree is isolated in a third branch. The tree of the amino acid concatenated sequences presented a similar pattern (Fig. 8), although with a rearrangement within the older sequences branch ([SC/15/2007, GM/2007, Med, SC/2007/Med]; [SC/1990/Med]).

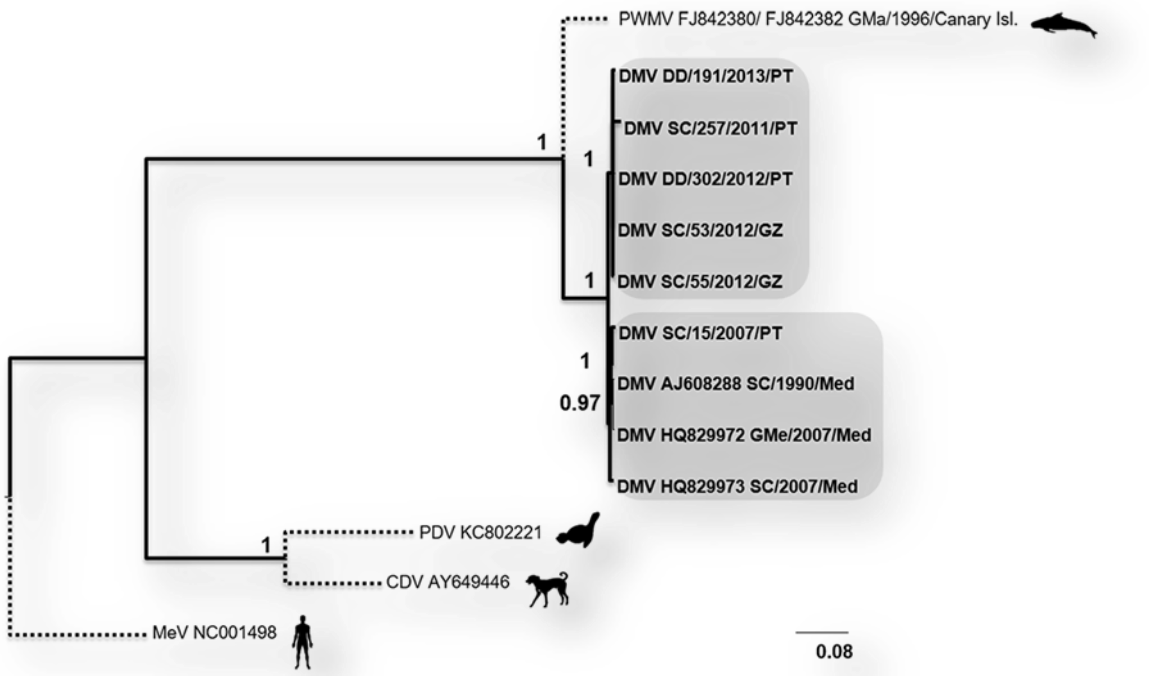


Figure 7 - Phylogenetic tree generated with concatenated nucleotide sequences alignment, inferred by Bayesian methods.

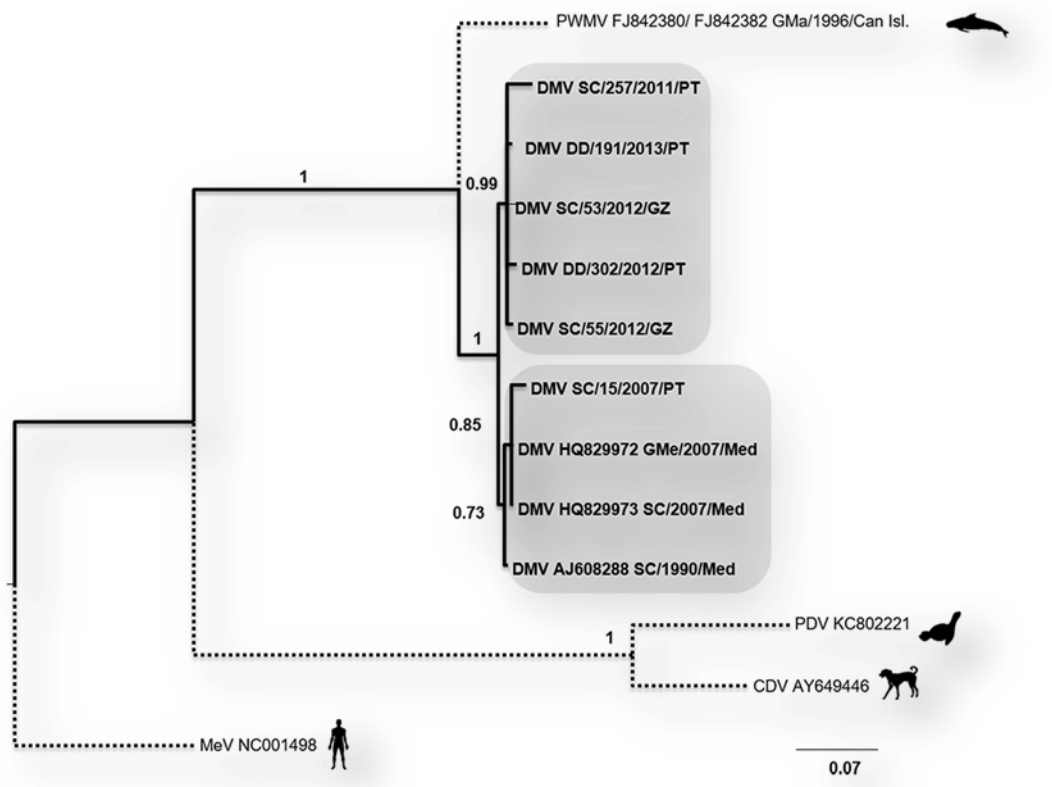


Figure 8 - Phylogenetic tree generated with the concatenated amino acid sequences alignment, inferred by Bayesian methods.

In the nucleotide tree for the F gene (Supplemental file 1) additional available sequences from the early nineties were included. Samples collected in the Atlantic during the 2011-2013 period clustered in the same branch; samples from the nineties clustered in a separate branch, and samples from the Mediterranean from 2007 clustered in a third branch, together with the sample SC/15/2007, similarly to the distribution of the concatenated trees. The PWMV was included in a unique branch. All branches were supported with high PP values.

For the H gene nucleotide tree (Supplemental file 2), a higher number of sequences were included. A set of sequences (9) from Portugal and Galicia ranging from 2009 to 2014 clustered in the same branch, supported by a PP value of 0.98. The SC/15/2007 sequence still clustered with Mediterranean samples from 2007 and samples from the early nineties were grouped in a separate branch. The new sequence for PMV included in this tree, branches out from the DMV samples, similarly to the PWMV sequence (PP value of 0.9).

The nucleotide tree for the P gene (Fig. 9) contained the higher number of sequences (35). One sequence from a guiana dolphin (*Sotalia guianensis*) collected in 2010 in Brazil

appeared to be a distinct strain from the already characterized strains of CeMV (PMV, PWMV and DMV. The two PWMV samples clustered in the same branch and the only PMV included in the tree was isolated from all the other sequences. All these strains were supported by high PP values. The DMV sequences included in this tree were all similar, including sequences from distinct geographic origins, such as Germany, Taiwan or the Mediterranean. Two sequences obtained from white-beaked dolphins in Germany and the Netherlands in different years (2007 and 2011 respectively) clustered together with a PP value of 0.99. Sequence AJ608288 from a striped dolphin collected in 1990 in the Mediterranean and sequence AF333347 from a pigmy sperm whale from Taiwan collected in 2001 also clustered together (PP 0.79). Samples from the Canary Islands collected in 2005, 2007 and 2009 clustered with samples from the Mediterranean (2007 and 2011), one sample from New Caledonia and one sample from Portugal (SC/15/2007). The remaining Portuguese and Galician samples clustered in the same clade with two samples with a different origin (KJ139454, Canary Islands and KC572861, Mediterranean).

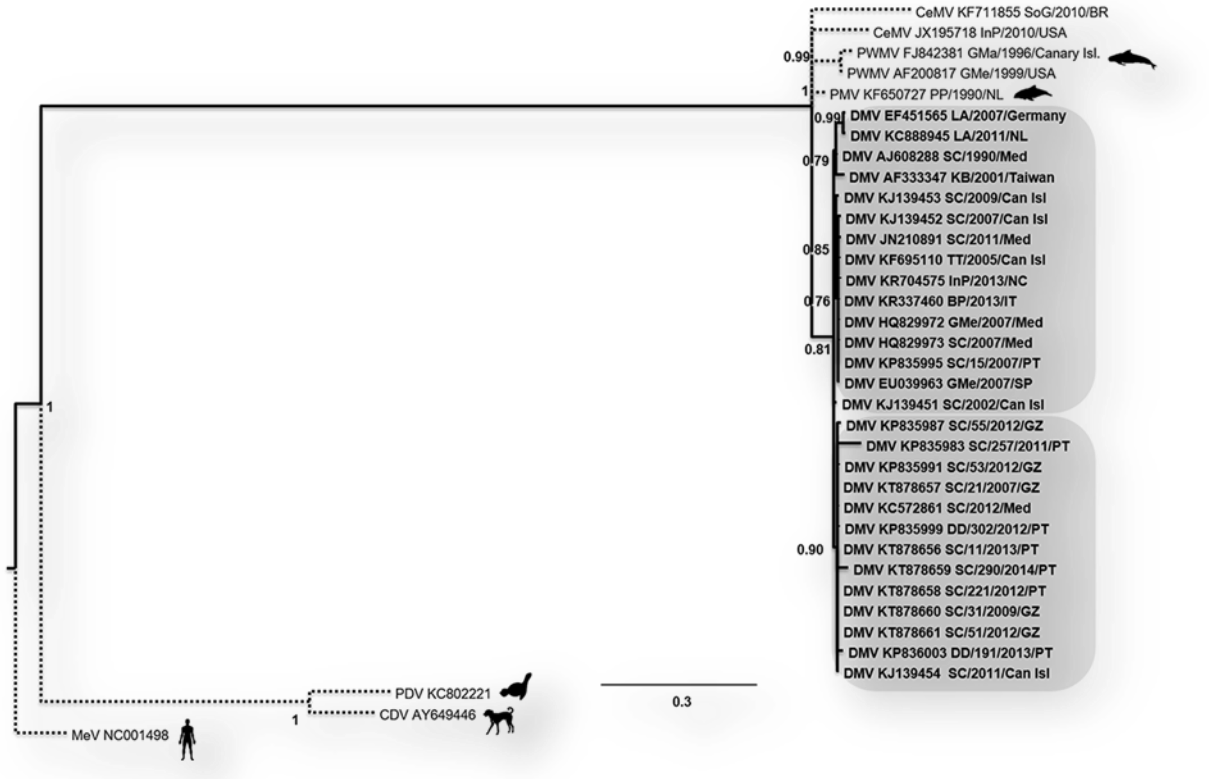


Figure 9 - Phylogenetic tree for the P gene nucleotidic sequences.

The N gene nucleotide tree (Supplemental file 3) showed a dislocation of sequences between branches. One branch included Atlantic samples from 2011 to 2013 grouped with the SC/15/2007 sequence and with sequences from the Mediterranean (1990 and 2007);

Atlantic sequences also from 2012 to 2014, were grouped separately. The remaining PWMV and PMV sequences appeared as two different outgroups.

4. Discussion

In this study we surveyed 279 animals and our results indicate a higher prevalence of DMV among stranded striped dolphins (20.6 %) when compared to stranded common dolphins (1 %) from the Atlantic based populations. Similar results had been previously described in the Mediterranean during the 1990–92 and 2006–08 CeMV breakouts, when striped dolphins presented higher death and stranding rates than other species (Bellière et al., 2011; Rubio-Guerri et al., 2013; Van Bressem et al., 2014). Several theories have been hypothesized for this higher mortality rate amongst striped dolphins in the Mediterranean: they were the most numerous species in the Mediterranean and serological studies suggested that, prior to the 2006–08 outbreak, antibody levels were low in this population rendering them more susceptible to the CeMV infection (Van Bressem et al., 2001); also, the fact that they are highly gregarious and tend to live in large pods could contribute to the spread of CeMV infection (Valsecchi et al 2004); high polychlorinated biphenyl (PCB) levels were also detected in the affected animals, leading to the hypothesis that an impaired immune system might have facilitated the infection by CeMV; finally, genetic susceptibility as a result of inbreeding in the Mediterranean population (Valsecchi et al. 2004), which had already been reported as relatively isolated from the Atlantic populations (Bourret et al. 2007).

Prevalence among striped dolphins from Galicia was 24.2 % while prevalence in striped dolphins stranded in Portugal was 16.7 %. Although this difference was not statistically significant, it is important to highlight that prevalence among striped dolphin samples from Galicia was probably underestimated since only lung samples were tested. Samples from the Portuguese coastline allowed testing several organs and antigen was only detected in brain samples of four individuals out of the 6 positive striped dolphins. It is therefore possible that the prevalence in striped dolphins from Galicia is being strongly underestimated. Previous studies from the Atlantic based populations were performed in the western part of the Atlantic, along the USA coast, and bottlenose dolphins were the most affected cetaceans in that area. In the Canary Islands a retrospective study was published in 2014 and 6 animals were positive for CeMV (5 striped dolphins and 1 common dolphin) (Sierra et al., 2014). In this study striped dolphins seem to be the most affected species sampled from the East Atlantic.

In four animals it was not possible to amplify viral genomic fragments by conventional RT-PCR. These samples recorded high CT values in the RT-qPCR, corresponding to a low target copy number (ranging from 105 to 943 copies), which would present a downside using a less sensitive conventional assay. Also, three of the four samples were collected in animals from Galicia originally stored at −20 °C, which may possibly imply RNA degradation hampering the amplification of longer genomic fragments, by conventional RT-PCR.

The genetic distances between samples were low among all sequences included in the phylogenetic trees. Nonetheless, PP values were high and consistent in all trees particularly in the DNA concatenated tree, adding robustness to the phylogenetic arrangement.

In the phylogenetic trees for the concatenated nucleotides the grouping of viral sequences followed a temporal arrangement, with samples collected since 2007 forming different clades. When a higher number of sequences was added to the trees (P gene tree) a phylogeographic arrangement becomes clear: all samples from Portugal and Galicia cluster together (with isolates ranging from 2007 to 2014), further away from the samples from the Mediterranean. The only exception seems to be the sequence from the animal SC/15/2007,clustering with samples from the Mediterranean, as well as with samples from the Canary Islands. Even samples from animals stranded in the south of Portugal (Algarve), such as SC/11/2013, clustered separately from samples obtained in the Mediterranean. This suggests that these populations may be relatively isolated from each other, which is supported by previous findings by other authors (Bourret et al., 2007). It is worth noticing that only one sample from a striped dolphin collected in the Canary Islands clusters closer to the Portuguese and Galician samples. All the other samples from the Canary Islands are closer to Mediterranean samples.

Positive samples for DMV antigen were detected annually since 2007 to 2013, showing that the virus is circulating in cetacean populations from the Atlantic off the coast of Portugal and northern Spain and both striped dolphins and common dolphins were found to be positive to viral infection. The infection was mapped in the available organs and positive lung samples were detected without association to higher mortality or stranding rates. Further studies would be necessary to determine if these animals had an acute, sub-acute or chronic infection and if the DMV infection was the cause of death. Animals DD/191/2013, DD/302/2012, SC/21/2007, SC/51/2012, SC/53/2012, SC/11/2013 and SC/257/2011 stranded alive and were in general emaciated and with high parasite loads, suggesting a sub-acute or chronic systemic infection. Histological and immunohistochemical studies should be performed to further characterize the necropsy findings. Four animals (SC) were positive only in brain samples, which might imply the development of chronic localized encephalitis after a systemic infection.

The two common dolphins positive for viral antigen (DD/302/2012 and DD/191/2013) were both alive at the time of stranding and presented high parasite loads and poor body condition. Animals DD/302/2012 and SC/221/2012 are also positive for cetacean gamma herpesvirus (Bento et al., 2019) with viral antigen detected systemically. Unlike Mediterranean populations of striped dolphin (Van Bressem et al., 2014), morbillivirus infection seems to be endemic in the population of striped dolphins from the Atlantic. This correlates to the serological survey conducted in 2011 in which 21.6 % ($n = 37$) of the analysed cetaceans cross reacted with Canine Distemper Virus antigen in a commercially available ELISA kit (unpublished observations Bento, C.). To date, the harbour porpoise was reported as the most affected species with morbillivirus infection in the north-eastern Atlantic, although infection is probably not endemic considering porpoises' solitary behavior (Van Bressem et al., 2014). Large populations are needed to maintain morbillivirus infections as endemic (Van Bressem et al., 2001) and although striped dolphin abundance has increased over the last years in the Portuguese Continental coast it is still a rather small population if compared to the common dolphin population (Araújo, H. personal communication). Notwithstanding, evidence suggests an endemic situation rather than an epidemic, since no outbreaks have been detected in the striped dolphin population of the Atlantic. Moreover, positive samples have been detected annually since 2007, indicating that this virus is actively circulating in this population reaching prevalence values as high as 24 % in the Galician samples. In 1999, dolphins stranded along the Atlantic coast of Spain had low antibody titers for CeMV. Considering the results obtained in this study, further serological studies are needed to deepen the knowledge about the epidemiology of this disease in striped dolphins.

Unlike striped dolphins, the prevalence of stranded common dolphins positive for viral antigen is much lower (1 %). The difference in CeMV prevalence between stranded common and striped dolphins needs to be fully assessed and further studies are needed to clarify the virus impact on cetacean populations and why do striped dolphins appear to be more susceptible to DMV infection. New approaches should be considered: viral enrichment and random amplification techniques associated with next generation sequencing could contribute to deepen the knowledge on this virus and its interaction with other pathogens.

Surveys are a unique tool to provide information on viral epidemiology, especially in free-ranging cetaceans.

5. Conclusion

Our results suggest that DMV infection is endemic in striped dolphin populations of the Eastern Atlantic. Since it was first reported in cetaceans in the early nineties subtle but consistent changes in the reported viral sequences suggest that the Atlantic and the Mediterranean populations are relatively isolated from each other, as suggested by other authors. The prevalence of infection in stranded common dolphins is very low when compared to striped dolphins, and our results are in agreement with previous reports that point to a higher susceptibility of striped dolphins to CeMV. Reasons for differences in susceptibility to this viral infection in different species should be further investigated and serological surveys should also be performed to assess their protection level towards CeMV infection.

CHAPTER III

Herpesvirus infection in marine mammals: A retrospective molecular survey of stranded cetaceans in the Portuguese coastline.

M.C. Bento, R. Canha, C. Eira, J. Vingada, L. Nicolau, M. Ferreira, M. Domingo, L. Tavares, A. Duarte Infection, Genetics and Evolution, Volume 67, 2019, Pages 222-233, ISSN 1567-1348, https://doi.org/10.1016/j.meegid.2018.11.013.

Abstract

Herpesvirus (HVs) infection has already been reported in cetaceans, but available information on its epidemiology is scarce. In this study we surveyed a total of 179 cetaceans belonging to 6 different species. Samples were obtained from cetaceans stranded along the Portuguese coastline, belonging to populations that roam the north-east region of the Atlantic Ocean. Detection of HVs was performed by conventional nested PCR. Amplicons were sequenced by Sanger's method and sequences used to construct phylogenetic trees by Maximum Likelihood method. Our results show that prevalence of positive samples, among fresh carcasses, reached 14.3% (10/70) and both alpha and gammaherpesvirus were detected. Histopathology showed that herpesvirus infection varied from absence of signs compatible with disease, localized genital lesions and systemic disease. Phylogenetic analysis revealed three clusters within the alphaherpesvirus family; within the gammaherpesvirus no subdivision was detected. All clusters included animals from different species and geographic origins. In seven of the positive HVs samples, co-infections with other agents such as morbillivirus and toxoplasma gondii were detected. The viral nucleotide sequences were not assigned to a specific animal species, nor presented a given geographic distribution, which may imply a wider distribution of herpesvirus in these animal populations. Our results are also the first report of herpesvirus infection in common dolphins (Delphinus delphis), with both alpha and gammaherpesvirus detected.

1. Introduction

Herpesviruses are responsible for a wide range of diseases and the course of infection can vary from subclinical to fatal infections with localized or systemic lesions (Saliki et al. 2006; Davison 2008). Their most unique feature is the persistent infection interleaved with periodic or continuous shedding (MacLachlan and Dubovi 2011a). The viral genome can remain latent, either as an episome or integrated in the host genome (provirus). Upon viral genome reactivation, often promoted by stress cellular signals, lytic viral replication takes place in the nucleus of the infected cell during the active stage of infection. The family Herpesviridae comprises three subfamilies: Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae (ICTV, 2011).

The characteristic appearance of HVs was initially used as an identification method to assign them to the taxonomic family *Herpesviridae*. Later, they were allocated to each subfamily by biological criteria (McGeoch et al. 2006). With the advent of molecular biology techniques such as the polymerase chain reaction (PCR), genetic criteria allowed the characterization of these viruses and their taxonomic assignment.

Research in this area rapidly evolved, due to the amount of HVs described: by 1996 around 100 distinct HVs had been described (VanDevanter et al. 1996); 120 in 2002 (Davison 2002) and, most recently, over 200 HV have been reported (Pellett and Roizman 2013). The growing number of known HVs sequences is mostly due to the development of a pan-herpesvirus detection system (VanDevanter et al. 1996). This system was designed to target the conserved motifs on the viral DNA polymerase (Dpol) using degenerate primers, allowing the amplification of a vast array of distinct HVs from different host species. This approach has allowed the successful investigation of the presence of HVs in different species, from pigeons and horses (Ehlers et al. 1999) to reptiles (Wellehan et al. 2003) and bats (Molnár et al. 2008), allowing the virus identification and the taxonomic assignment to a subfamily (α, γ or β).

In marine mammals, HVs were first reported in harbor seals (Phocine herpesvirus – 1 [PhHV-1]) by electron microscopy and serum neutralization assays (Osterhaus et al. 1985). Since then, HVs have been reported in several species of pinnipeds (Harder et al. 1996; Lipscomb et al. 2000; Goldstein, Lowenstine, et al. 2006; Goldstein et al. 2006). In odontocete cetaceans HVs have already been detected in Monodontidae (e.g. belugas, Martineau et al., 1988), Phocoenidae (porpoises, Kennedy et al 1992), Delphinidae (dusky dolphins and striped dolphins, Bellière et al., 2010; Van Bressem, Van Waerebeek et al 1994) and Ziphiidae, (beaked whales, (Arbelo et al. 2010; Arbelo et al. 2012). In Mysticetes, HVs have been recently reported in two species from the Balaenoptera genus (Melero et al. 2015).

With respect to cetaceans, viruses capable of causing disease and immunosuppression, such as morbilliviruses, were detected in animals with concurrent herpesvirus infection (Bellière et al. 2010). Also, papillomaviruses were detected in herpesvirus positive animals (Cruz et al. 2014). So far, cetacean HVs were included in the *Alphaherpesvirinae* or *Gammaherpesvirinae* subfamilies. Alphaherpesviruses have been associated with fatal disseminated infections (Blanchard et al. 2001), localized genital lesions (Benson et al. 2006), proliferative dermatitis (Manire et al. 2006), tubule-interstitial nephritis (Arbelo et al. 2012), among other infections (Arbelo et al. 2010; Soto et al. 2012; Sierra et al. 2014). Gammaherpesviruses have been detected in cetaceans associated to cutaneous and mucosal lesions (Saliki et al. 2006; Benson et al. 2006)

2. Theory

So far, no information on herpesvirus infection was available for samples obtained in Portugal. Scientific reports of herpesvirus infection in several species of cetaceans on samples obtained in Spain are available, but mostly from samples from the Canary Islands and Mediterranean (Arbelo et al., 2012, 2010; Melero et al., 2015; Sierra et al., 2015). No information is available from the populations that roam the Atlantic waters of the Iberian Peninsula.

Previous work identified the presence of morbillivirus infection in samples from the Portuguese coastline (Bento et al. 2016). Integrating this information with a survey of herpesvirus will allow us to deepen the knowledge of the health status of these populations.

The present study aims at identifying novel or known herpesviruses in samples from cetaceans stranded on the Portuguese coastline. Phylogenetic relations between these new herpesviral sequences and representatives of the known Herpesviridae subfamilies and genera will be explored through Maximum Likelihood (ML) analyses. In available samples, histopathology will be performed in order to identify lesions compatible with herpesviral infection, and to assess cause of death.

3. Materials and Methods

3.1 Sampling

The animals stranded along the coast of Portugal between 2011 and 2014 and were accessed by experienced personnel belonging to the Portuguese stranding network, coordinated by the Institute for Nature Conservation and Forests (ICNF) and Sociedade Portuguesa de Vida Selvagem (SPVS) (Fig. 10).

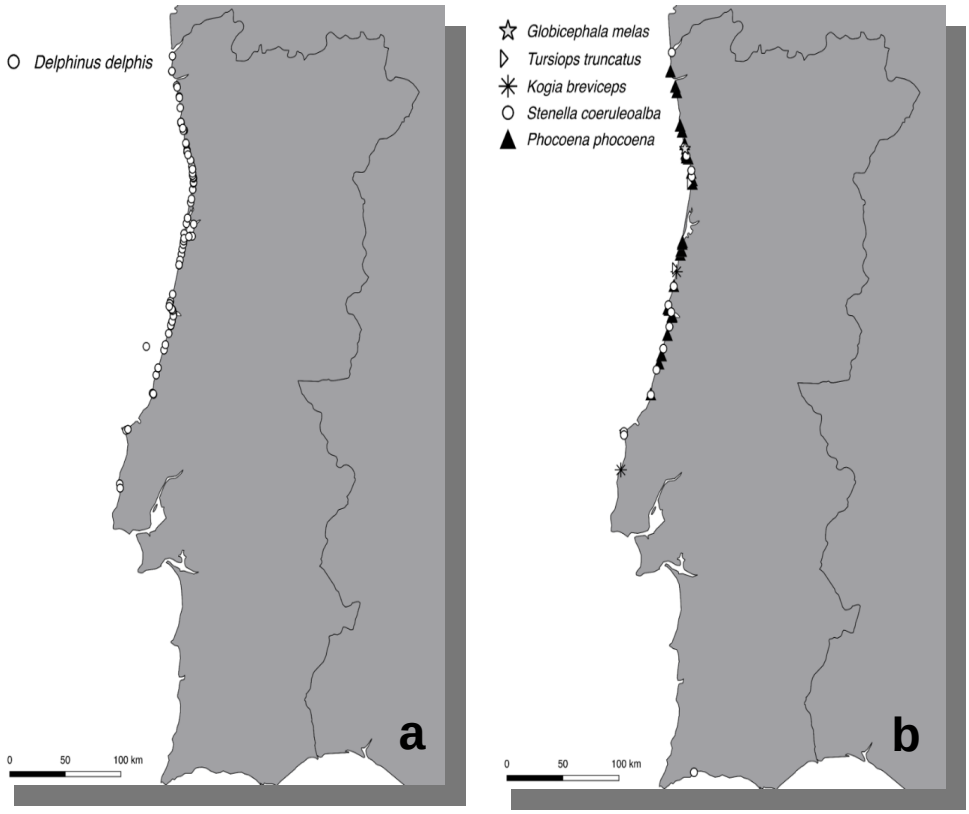


Figure 10 - Stranding sites for common dolphins (a) and other species (b) analyzed for herpesviral infection.

Detailed necropsies were performed, depending on the carcasses' preservation status (decomposition code) (Geraci and Lounsbury 1993) and the cause of death was determined when possible. Data and samples were collected according to standard protocols (Kuiken and Hartmann, 1991) and each animal was identified using an alphanumeric code composed of the initials for the species, an attributed number, and the year of stranding. Samples were kept in 2 mL vials, submerged in RNALater (Sigma-Aldrich, St. Louis, MO, USA) and stored at −20 °C, until the molecular analysis. For histology, tissue samples were stored in 10% formalin. All the technicians that work on marine animal strandings from the Portuguese Wildlife Society are licensed for capture, handling, tagging and sample collection in mainland Portugal under the Decree-Law n° 140/99 of 24th April, with new redaction given by Decree-Law n° 49/2005 of 24th February and Decree-Law n° 316/89 of 22nd September. The licenses are issued by the Institute of Nature Conservation and Forests. These licenses

are issued every year. Also, The Portuguese Wildlife Society, which holds the Marine Animal Tissue Bank, is registered in CITES as a scientific institution with code PT009. Data and samples were collected according to standard protocols (Kuiken and Hartmann, 1991) and each animal was identified using an alphanumeric code composed of the initials for the species, an attributed number, and the year of stranding.

A total of 179 stranded cetaceans belonging to 6 different species were tested for the presence of herpesvirus DNA (supplemental file 4): 126 common dolphins (Delphinus delphis), 31 harbor porpoises (Phocoena phocoena), 17 striped dolphins (Stenella coeruleoalba), 2 bottlenose dolphins (Tursiops truncatus), 1 long-finned pilot whale (*Globicephala melas*) and 2 pygmy sperm whales (Kogia breviceps). Samples identification included an alphanumeric code composed of the initials for the species, an attributed number, and the year of stranding. Of the animals tested 76 were males and 100 females. In three animals the sex was not registered. Most samples were from juvenile animals (115), while only 52 were adult animals. Animals less than one year old summed to a total of 11 animals. In one animal, age class was not estimated.

3.2 DNA extraction

For each tested animal, DNA was extracted from a pool of organ tissue containing, when available, liver, kidney, lung and brain samples. If present, samples of genital lesions were also included in the pool. Total DNA was extracted using DNeasy Blood & Tissue Kit (QIAGEN, Dusseldorf, Germany), according to the manufacturer's protocol. DNA concentration was measured with Nanodrop 2000C (Thermo Scientific, Whaltam, MA, USA), and stored at −20 °C.

3.3 DNA amplification

Presence of herpesvirus DNA in extracted total DNA was detected by PCR, in a nested format, using five degenerate primers (VanDevanter et al. 1996). This technique targets a conserved region within the herpesvirus polymerase gene, with an expected amplicon size ranging from 215 bp to 315 bp.

In the first PCR reaction, 100 ng of extracted total DNA were mixed with 5 PRIME MasterMix (5PRIME GmbH, Hilden, Germany) (1.6 units of Taq DNA polymerase; 250 µM of each dNTP; 2.0 mM of Mg2+) and 400 nM of each primer (DFA, ILK and TGV) in a final

volume of 25 µL. The PCR product obtained in the first reaction was used as a template for the second round PCR (2.5 µL), using the same mastermix and 400 nM of primers TGV and IYG.

Cycling conditions for both PCR reactions included an initial incubation at 94 °C for 5 min, followed by 45 cycles of amplification: 94 °C for 30 s, 46 °C for 1 min and 72 °C for 1 min. A final elongation step was performed at 72 °C for 7 min and kept at 4 °C.

The PCR reaction products were resolved by electrophoresis in 1.5% agarose gels, stained with 500 nM of GelRed (Biotium, Hayward, CA, USA). If amplicons of the expected size were found, the PCR reaction was purified with DNA Clean & Concentrator-5 (Zymo Research, Irvine, CA, USA), according to the manufacturer's protocol, and eluted in a final volume of 10 µL.

In positive samples, all the tissues available, including those in the initial tissue pool were subsequently tested separately, to map the HV infection. The amplified DNA was directly sequenced with the second round TGV and IYG primers by Sanger's method (STABVida, Portugal).

3.4 Phylogenetic Analysis

The identity of the nucleotide sequences was confirmed by a nucleotide BLASTn suite (Altschul et al. 1990) of the Genbank database, with the default settings.

The sequences obtained in this study were imported to Unipro UGene (Okonechnikov et al. 2012), where the primer regions were excised. The resulting sequences were translated to the deduced amino acid sequences and all positions containing gaps and missing data were eliminated. Sequences were aligned using MUSCLE (Edgar 2004), with the program's default settings. This alignment was imported into MEGA 7.0.21 (Kumar et al. 2016) and a phylogenetic tree was constructed using the Maximum Likelihood Method and the JTT matrix-model (Jones et al. 1992) with a discrete Gamma distribution to model the evolutionary rate differences among sites (5 categories (+G, parameter = 0.6180)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 9.2593% sites).

To construct the phylogenetic trees, sequences were retrieved from Genebank. Sequences belonging to alphaherpesvirus (30 sequences), gammaherpesvirus (19 sequences) and one sequence of betaherpesvirus. One sequence of an Iguanid Herpesvius was used as an outlier in one of the trees. Accession numbers for all the sequences used are specified in the supplemental file 5. Amino acid sequences that were 100% identical and did

not add information to the phylogenetic tree were withdrawn from the alignment, and are identified within square brackets, in the phylogenetic tree.

3.5 Statistical Analysis

The Fisher's exact test (http://vassarstats.net/) was performed to test for significant differences in the number of HV positive animals in relation to carcass conservation condition and according to cause of death, with a significance level set to *p*-value ≤.05.

3.6 Histology

For histology, tissue samples were processed as routine, embedded in paraffin and 4 µm sections were stained with hematoxylin and eosin (H&E) in an automated stainer for light microscopic analysis.

Immunohistochemical staining for detection of Toxoplasma gondii and Morbillivirus antigen was performed using the DAKO PT-link system and the Autostainer-EnVision (DAKO), according to already established routine procedures. For each sample positive and negative controls were used. The primary antibody against *Toxoplasma gondii* was a rabbit polyclonal antibody at a dilution of 1:4000 (courtesy of Dr. Bjerkas). The antibody against Cetacean morbillivirus (CeMV) was a monoclonal commercial antibody (VMRD) against the nucleoprotein of the Canine Distemper Virus that cross-reacts with CeMV, used at 1:50000 with an incubation step of 40 min. After the washing steps, the secondary antibody was incubated (DAKO Rabbit/Mouse EnVision Detection System (Dako Ref.: K5007) for 40 min at room temperature, with the dilution recommended by the manufacturer. Slides were incubated for 5 min in DAB-Chromogen-hydrogen peroxide (Dako k3468) and stained with Mayer hematoxylin.

4. Results

4.1 Herpesvirus nucleic acid detection and phylogenetic analysis

Out of the 179 tested animals, 14 (7 males; 7 females) were positive to herpesvirus by conventional PCR, corresponding to a percentage of 7.8% of positive

animals (Table 7). Animals DD-105-2011 and DD-112-2011 were both positive for HV in the lung, the only available organ. Two different HV sequences were obtained from animal DD-317-2011. Two of the animals that were HV positive were also Dolphin Morbillivirus positive (SC-221-2012 and DD-302-2012) (Bento et al. 2016). Animal SC-189-2013 tested positive in the tissue pool, but not in the DNA extracted from the individual organs. Lung, kidney and liver were the organs most frequently tested. These were included in the pool of tissue samples that were used in the survey and were also tested individually in the HV positive pools. Lymph nodes and lesions were also tested in positive animals whenever they were available, and all the lymph nodes tested in the positive samples were positive, except for animal DD-230-2012 and DD-297-2011.

Table 7 – Tested and positive samples for herpesvirus antigen detected by conventional nested-PCR.

	Pool	Liver	Kidney	Lung	Brain	Spleen	Regional lymph nodes			Genital lesion
							Pulmonary	Mesenteric	Pre-escapular	
DD-105-2011				X						
DD-112-2011				X						
DD-132-2011	X	X	X							
DD-141-2011	X		X							
DD-183-2011	X	X	X							
PP-273-2011	X	X	X	X			X			
DD-297-2011	X	X								
DD-317-2011	X		X	X				X	X	
DD-206-2012	X		X	X			X			
SC-221-2012	X		X	X			X			
DD-230-2012	X	X								X
DD-302-2012	X	X	X	X	X		X			
SC-189-2013	X									
PP-271-2013	X				X					

Grey cells indicate the tested samples; positive samples are marked with an X.

The number of positive samples was analyzed according to the cause of death: animals were divided into 2 categories; bycaught animals (11/128) and animals that died from disease or where cause of death was not determined (3/37). No association between cause of death and positive HV results were detected ($P = 1$).

The conservation score of the carcasses was also analyzed. Conservation scores range from 1 to 5, 1 being animals that were stranded alive, and 5 being the more decomposed carcasses. There was a significantly higher number of positive HV animals among fresh animals (conservation scores 1 and 2; $n = 70$) in comparison to the group of samples from the decomposed animals (conservation score 3 and 4; $n = 109$) ($P = 0{,}024$).

When considering samples from fresh carcasses only, the percentage of HV positive animals increases up to 14.3% (10/70).

Sequences obtained in this study were uploaded to an online database (Genbank) and are available online with sequential accession numbers from MG437203 to MG437217.

4.2 Phylogenetic Analysis

To clarify the taxonomic assignment of the HV sequences a tree was constructed (Fig. 11) with sequences from the Alphaherpesvirinae, Betaherpesvirinae and *Gammaherpervirinae* subfamilies. For the *Alphaherpesvirinae* subfamily, four different genera were chosen: Simplexvirus (Herpes simplex virus type 1; Eidolon helvum simplexvirus 1); Varicellovirus (Equid herpesvirus 1; Suid herpesvirus 1); Mardivirus (Collumbid herpesvirus 1) and Iltovirus (Passerid Herpesvirus 1). For the subfamily *Betaherpesvirinae,* one sequence of Human herpesvirus 6A was included, and to represent the *Gammaherpervirinae* subfamily, a sequence of Human herpesvirus 4. Within the Alphaherpesvirus the two *Simplexvirus* sequences were grouped together (BS 91%) as well as the two *Varicellovirus* sequences, despite the lower bootstrap value of this cluster (BS 44%). The *Iltovirus* and *Mardivirus* sequences were isolated in separate branches. The Portuguese HV sequences belonging to the *Alphaherpesvirinae* subfamily were distributed in three separate branches, without clustering in either of the HV genera. Sequences DD317/2011b and SC221/2012 clustered together (α1) with a high bootstrap value (BS 100). Sequences DD317/2011a; PP273/2011; DD302/2012; DD206/2012; DD297/2011 clustered in the same branch (α2) although supported by a lower bootstrap value (49%). The α3 branch included sequences DD141/2011, DD132/2011, PP271/2013 and DD112/2011 (BS 89%). In the Gammaherpesvirinae branch (BS 86%), the inclusion of the HHV4 (AJ507799) sequences did not resolve the four Portuguese HV sequences (SC189/2013; DD183/2011; DD230/2012 and DD105/2011) positioning. No Portuguese HV sequences clustered within the *Betaherpesvirinae* branch.

The evolutionary history was inferred using the ML method by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT + G + I model, and then selecting the topology with superior log likelihood value (Jones et al. 1992). Trees were drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

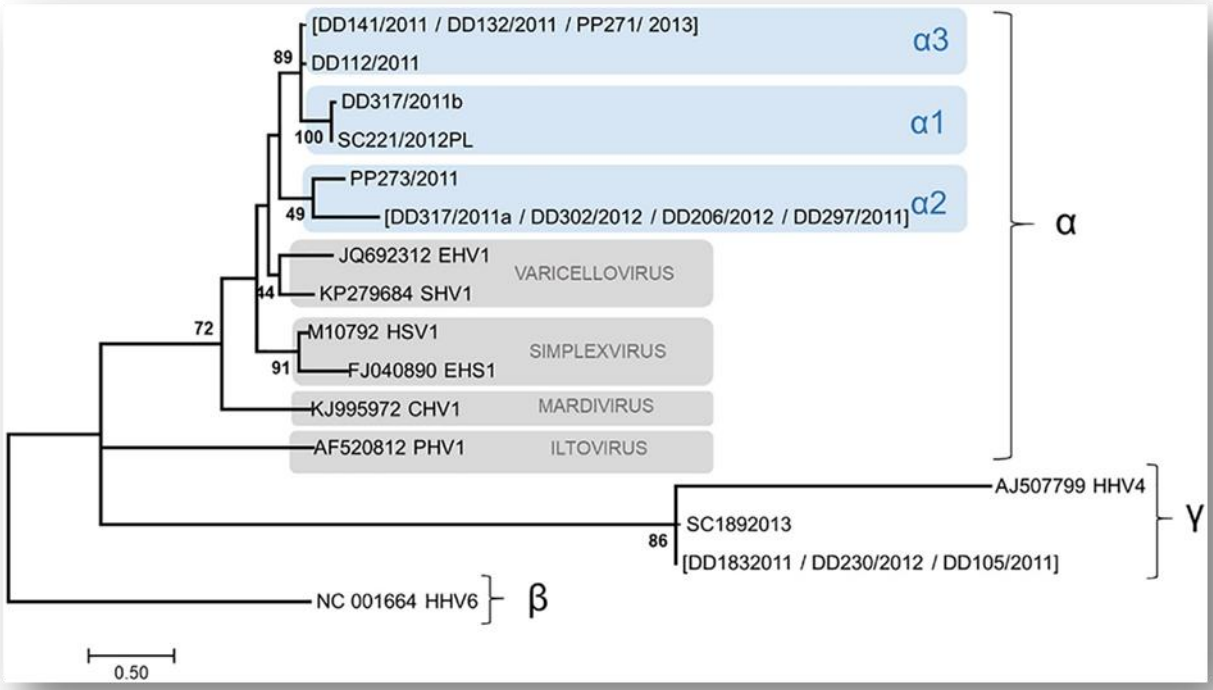


Figure 11 - Molecular Phylogenetic analysis of Portuguese sequences, subfamily alpha gamma and betaherpesvirus, with alphaherpesvirus genera represented.

A phylogenetic analysis was performed including the available HV sequences detected in marine mammals and previously characterized. In this tree, (Fig. 12) the Portuguese HVs sequences obtained in the present study form two distinct branches supported by high bootstrap (BS) values, corresponding to the subfamilies *Alphaherpesvirinae* and *Gammaherpesvirinae*. In one branch with 78% BS, sequences previously identified as alphaherpesvirus and the Portuguese samples: DD-112/2011, DD-132/2011, DD-141/2011, PP-273/2011, DD-297/2011, DD-317/2011a, DD-317/2011b, DD-206/2012, SC-221/2012, DD-302/2012 and PP-271/2013, clustered together. In the other branch with a 100% BS, all sequences previously identified as gammaherpesvirus clustered

with the Portuguese samples: PP-105/2011, DD-112/2011, DD-183/2011, DD-230/2012 and SC-189/2013. Gammaherpesvirus represented in the tree belong to two Blainville's beaked whales; one porpoise; a Risso's dolphin; two minke whales; ten bottlenose dolphins; a striped dolphin and a dwarf sperm whale.

The *Alphaherpesvirinae* subfamily sequences were further divided into three different monophyletic branches; α-1, α-2 and α-3; each supported by high BS values (86, 77 and 80% respectively). Each group included HV sequences amplified from animals belonging to different species. The α-1 group included sequences from a Cuvier's beaked whale, an orca, and bottlenose and striped dolphins, together with the Portuguese HV sequences from a common dolphin (DD-371/2011b) and a striped dolphin (SC-221/2012). The α-2 group included sequences from a Blainville's beaked whale, a fin whale, a melon headed whale, bottlenose, striped dolphins and the Portuguese sequences of a porpoise (PP-273/2011) and common dolphins: DD-317/2011a; DD-302/2012; DD-206/2012; DD-297/2011. In the α-3 group, sequences from a false killer whale, bottlenose and striped dolphins grouped with the Portuguese common dolphin sequences DD-112/2011, DD-141/2011 and DD-132/2011 and a porpoise sequence (PP-271/2013).

The evolutionary history was inferred using the ML method by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT + G + I model, and then selecting the topology with higher log likelihood value (Jones et al. 1992). Trees were drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016). Portuguese sequences are marked with an asterisk.

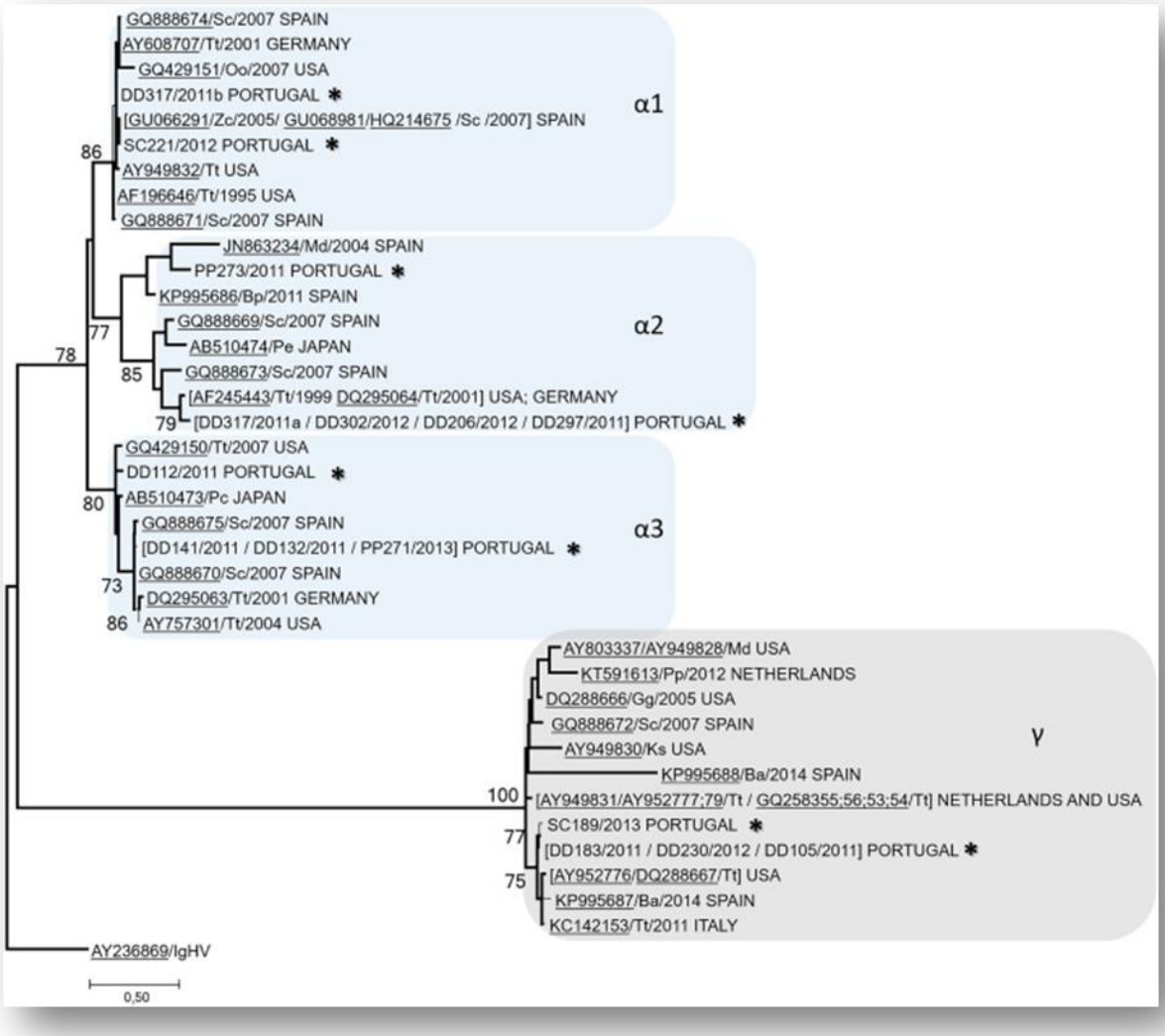


Figure 12 - Molecular Phylogenetic analysis of Portuguese sequences and sequences previously detected in marine mammals.

4.3 Gross and microscopical findings

Histopathological examination was performed in seven positive animals: SC-221/2012, DD-302/2012, DD-317/2011, DD-112/2011, DD-230/2012, SC-189/2013, and PP-271/2013. Stranding condition, relevant findings and cause of death are summarized in table 8.

Table 8 - Stranding condition, body condition, most significant findings (gross and microscopic), virology results and cause of death for animals in which histopathology was performed.

ID CODE	STRANDING CONDITION	BODY CONDITION	GROSS AND MICROSCOPIC FINDINGS	VIROLOGY	CAUSE OF DEATH
SC-221/2012	Live Stranding	Poor	Ectoparasites and ulcerations in the mouth, pneumonia, encephalitis, hepatitis and lymphoid depletion with necrosis: consistent with HV infection	+ Hv; + CeMV	Pneumonia/encephalitis
DD-302/2012	Live Stranding	Poor	Active toxoplamosis with hepatitis and adrenalitis. Generalized lymphoid depletion.	+ Hv; + CeMV	Toxoplasmosis
DD-317/2011	Live Stranding	Poor	Bronchointersticial pneumonia, most likely of viral origin	+HV	Pneumonia
DD-112/2011	Found dead	NR	No lesions reported; aspiration of foreign material.	+HV	Not determined
DD-230/2012	Live Stranding	Good	Parasitic gastritis with ulcers in the first stomach and a moderate to heavy load of nematodes	+HV	Bycatch
SC-189/2013	Live Stranding	Poor	Heavy load of ectoparasites and extensive pox-like lesions in the skin	+HV	Euthanasia
PP-271/2013	Found dead	Good	Severe necrotizing hemorrhagic gastritis in the second stomach	+HV	Bycatch/ gastritis

Animal SC-221/2012 was a live stranding that died during rescue efforts at the beach. The animal presented ectoparasites and ulcerations in the mouth and a poor body condition. It tested positive both for CeMV (Bento et al. 2016) and HV nucleic acid, although it was negative in the immunohistochemistry (IHQ) for CeMV in the lung, diaphragmatic lymph node, spleen and central nervous system. In the histological findings, the animal showed purulent catarrhal bronchopneumonia and lymphoid depletion with necrosis of the lymphoid organs. Giant syncytial cells with basophilic intranuclear inclusion bodies were detected in the lymph nodes and lungs. Furthermore, this animal presented a light focal mononuclear encephalitis, a mononuclear interstitial (periportal) hepatitis and a parasitic gastritis in the second and third stomachs. Lesions in the lymphoid organs and central nervous system as well as the morphology of the observed inclusion bodies were consistent with herpesvirus infection.

Animal DD-302/2012, similarly to animal SC-221/2012, was also a live stranding and was positive for both CeMV and HV nucleic acids by molecular detection, and negative in the IHQ for CeMV in the lung, diaphragmatic lymph node, thyroids and central nervous system. The histological findings included a generalized lymphoid depletion with amyloid deposition in the lymph nodes; additionally, an active toxoplasmosis was detected, with lesions in the liver and adrenal glands, including a necrotizing lymphoplasmacytic hepatitis and a

necrotizing adrenalitis. No *Toxoplasma gondii* infection was detected in the CNS or lymph nodes.

Animal DD-317/2011 stranded alive, died 48 h after rescue and transport to the rehabilitation center. A subacute bronchointersticial pneumonia was identified as well as apoptotic lesions in the liver, most likely due to hypoxia or toxicity. Pneumonia was the most relevant lesion, most probably due to a primary viral infection followed by a subsequent bacterial infection. The liver lesions were mild to moderate and could have been a result of the stranding process.

Animal DD-112/2011 did not show clear signs of disease and the only relevant observation in histology was the presence of foreign material in the lungs (muscle fibers with nucleated erythrocytes), probably due to agonal aspiration.

Animal DD-230/2012 stranded alive twice before being found dead at the beach. This animal presented a parasitic gastritis with ulcers in the first stomach and a moderate to heavy load of nematodes. It had a genital lesion that tested positive to herpesvirus and a lymphoplasmacytic urethritis was identified in the histological exam, with possible viral inclusion bodies in the ureteral epithelium. These lesions were unlikely to have caused death and this animal was in good body condition.

Animal SC-189/2013 was a live stranding that was euthanized. This animal had a heavy load of ectoparasites and extensive pox-like lesions in the skin and a mild purulent catarrhal bronchopneumonia. The thymus and lymph nodes had intra-nuclear acidophilic inclusion bodies in lymphoid cells and syncytial giant cells. It had a superficial necrotizing dermatitis with thrombi, perivascular infiltrates and corneal micro-pustules. In the areas with lesions, keratinocytes seem to have cytoplasmic inclusions; highly suggestive of a viral infection. Although this animal tested positive for HV nucleic acid in the tissue pool, it was not possible to map the infection.

Animal PP-271/2013 had signs of bycatch. Its histology presented a severe necrotizing hemorrhagic gastritis in the second stomach. Etiology was not determined, but it was possible that these lesions could be due to Clostridium spp., which could have caused a fatal toxemia.

Bycatch was the cause of death determined at necropsy for several animals: DD-183/2011, DD-230/2012 and DD-205/2011, all positive to gammaherpesvirus; and PP-273/2911, DD-206/2012, DD-297/2011, DD-141/2011 and DD-132/2011, positive to alphaherpesvirus.

5. Discussion

A systematic approach is necessary to implement health monitoring in wild marine mammals' populations. Routine collection of tissue samples from stranded animals contributes to the establishment of a tissue bank, providing available samples for health surveillance. Systematic surveys play an important role in epidemiological surveillance and allow the detection of emerging and resurgence of infectious diseases (Gilbert et al. 2011; Delwart 2012). In unusual mortality events (UME) or disease outbreaks, such surveys are even more critical for determining the cause of the mortality. Routine evaluations with established baselines and established laboratory working relationships are essential for such investigations.

In this work we surveyed 179 cetaceans stranded along the Portuguese coastline and found a total percentage of positive animals of 7.8% (14/179). Since carcass condition score was found to be related with HV DNA polymerase detection, while the percentage of HV positive animals rises to 14.3% (10/70) when considering only samples from fresh carcasses. This percentage is closer to values reported in similar studies using conventional PCR detection systems: 25.8% in chimpanzees (Seimon et al. 2015), 27% in rodents (Ehlers et al. 2007) and 26% in humans (Minjolle et al. 1999), although still inferior. Considering that, although the target viability is not necessary for PCR assays, maintenance of the biological matrix under optimum conditions for virus detection will enhance pathogen detection by these techniques. Therefore, samples should be correctly collected and maintained in order to improve antigen detection by molecular and serological assays (MacLachlan and Dubovi 2011b; Storch and Wang 2013). Considering our results, we recommend that all available samples should be tested to detect the highest number of sequences possible. However, if resources are scarce, we would recommend testing only fresh samples, especially when estimating disease prevalence. Otherwise, the results can be considerably underestimated.

Although herpesvirus in cetaceans have been documented since 1988 (Martineau et al. 1988), the available information is still scarce (Lecis et al. 2014). The pan-herpesvirus primers used in this work allowed the detection of herpesvirus in species with no previous reports of herpesvirus infection, such as the pacific walrus, fin whale, minke whale or even box turtles (Melero et al. 2014; Melero et al. 2015; Sim et al. 2015). Our work is the first time herpesvirus infection was reported in common dolphins, with both alpha and gammaherpesvirus detected. The sequences obtained in this species do not seem to be species-specific, similarly to what happens in the other species represented in the tree.

In cetaceans, there are still very few sequences available and more studies are needed to assess the diversity of herpesvirus in these species. Furthermore, few published studies focused on the prevalence of infection in cetaceans (Sierra et al. 2014; Elk et al.

2016) and this is one of the few studies that systematically tested stranded cetaceans for herpesvirus antigen by analyzing samples from 2011 to 2014. The development of specific primers for the amplification of cetacean herpesvirus, could provide a more robust molecular assay, allowing a more rapid and efficient detection of positive samples.

Concerning the phylogeny of the reported Portuguese HV sequences, and although the short Dpol sequence available allows only for the subfamily identification (Maness et al. 2011), the phylogenetic tree generated from the obtained and previously reported sequences displayed three distinct clusters within the Portuguese alpha-herpesvirus, circulating in Atlantic waters off the coast of Portugal. None of these Portuguese sequences clustered with any of the previously identified genera.

The Dpol region is highly conserved in all the HVs, making it the gene of choice for examining phylogenetic relationships, since homologous sites give a more reliable alignment (VanDevanter et al. 1996). However, it does not allow a more precise phylogenetic analysis (Ehlers et al. 2008), only achieved with the concatenated alignments of up to 8 core genes in mammalian HVs (McGeoch et al. 2006).

In one of the positive animals (DD-317/2011), two different sequences of herpesvirus were co-amplified. Although they were both included within the Alphaherpesvirinae cluster, one sequence grouped within the α-1 group (DD-317/2011b), while the other sequence grouped in the α-2 group (DD-317/2011a), sharing 59% of the nucleotide sequence identity. Co-infection with different viral strains was already identified. Bellière et al., 2010 reported the detection of HV sequences from more than one strain, in three of five dolphins (3/5; 60%) infected with both HV and CeMV. Later, Sierra et al., 2014 reported four new α-herpesvirus sequences, two of them detected in the same animal. Also, three different sequences of α-herpesvirus were detected in the skin, spleen and blood of a single bottlenose dolphin stranded in Germany (Benson et al. 2006).

In the present study, animals SC-221/2012 and DD-302/2012 were both co-infected with herpesvirus and morbillivirus and animal DD-302/2012 was also co-infected with Toxoplasma. The histological evaluation performed in 7 of the 14 positive animals showed that, although in some cases no lesions attributable to HV infection were found (e.g. animal DD-112/2011), in others there were clear signs of this viral infection. Animal SC-189-2013 had signs of a systemic viral infection, and animal DD-230/2012 showed localized lesions in the ureteral epithelium and an associated genital lesion, positive to HV. In both cases, the amplified HV sequences clustered with gammaherpesvirus sequences (Fig. 11 and 12). Gammaherpesvirinae can display very different pathophysiology's and so far in cetaceans they have only been found associated to skin and genital lesions (Benson et al. 2006; Lecis et al. 2014; van Beurden et al. 2015), which concurs with the macroscopic lesions observed in animal DD-230/2012. However, in other animal species gamma-

herpesvirus have been found associated to fatal systemic infections, such as the malignant catarrhal fever affecting ruminants (Russell et al 2009). In animal SC-189/2013 with signs of a systemic viral infection, we cannot exclude the presence of a different HV, justifying the differences between the histological evaluations, especially since we could not map the infection in the different organs.

In a previously published paper by Sierra et al., 2014, it was suggested that there could be a pathogenic branch of alpha-herpesviruses in cetaceans that could be responsible for fatal cases worldwide. In our study, animals SC-221/2012 and DD-317/2011 corroborate this hypothesis, with signs of viral disease severe enough to cause death.

6. Conclusion

Molecular detection of viral antigen in animals with no signs of disease is consistent with the epidemiology of herpesviruses that have the ability to remain latent for long periods of time and also reinforces the fact that these viruses most likely evolved with their hosts and are well adapted to them. Despite this fact, these viruses are also capable of causing severe disease, especially when associated to other pathogenic agents such as morbilliviruses and toxoplasmosis.

Our results suggest that herpesvirus infection plays an important role in cetacean morbidity. We detected herpesvirus in common dolphins, porpoises and striped dolphins. To our knowledge, this work is the first report of herpesvirus infection in common dolphins (*Delphinus delphis)* in the Atlantic Ocean; providing 11 new sequences of herpesvirus in this species. Although animals from different species were analyzed and compared to existing sequences, it is important to highlight that sequences from different species cluster together in the phylogenetic tree, pointing to the idea that these viruses are not species-specific.

The virus was detected in animals with severe systemic disease, particularly when associated with morbillivirus infection. Also, animals with mild localized lesion caused by herpesvirus infection were detected. Animals that showed moderate or severe systemic disease had alphaherpesvirus detected, while gammaherpesvirus was mostly found in mild or localized lesions.

This study allowed the detection and amplification of 15 new sequences of the conserved DNA polymerase of herpesvirus affecting cetaceans.

CHAPTER IV

Health assessment in common dolphins: disease associated shifts in cytokine mRNA expression.

M.C. Bento, P. Verdelho, C. Eira, J. Vingada, H. Araújo, M. Ferreira, L. Tavares, A. Duarte

Abstract

Cytokines play a key role in the establishment of an immune response and are crucial in host immunity during infection and inflammatory processes. Viral infections are capable of manipulating cytokine expression in order to favor viral success. Recognizing reference values for these cytokines in healthy animals is the first step to detecting immunological shifts caused by infection or stress. The availability of materials from free roaming cetaceans is a definite limitation towards establishing normal range values for biological parameters. Bycaught cetaceans are a good opportunity to obtain samples from individuals that died as a result of a rapid traumatic event, showing no signs of disease, which allow estimating baseline values for free roaming, healthy populations. By establishing a control group composed of healthy free-roaming common dolphins, we were able to determine baseline values for cytokine expression for IFN, IL4, IL6, IL10, IL12, TNF and IL1β. Cytokine profiles from animals positive for dolphin morbillivirus and *Toxoplasma gondii* were then compared with those of the control group, in order to determine the immunological shift promoted by these pathogens, especially considering morbillivirus' immunosuppressive nature. The relative expression of IFN, IL-4, TNF and IL1B was significantly higher in the healthy (control) group while IL-10 values were significantly overexpressed in the sick group when compared to the control group.

1. Introduction

Marine mammals are considered marine ecosystem sentinels, whose health reflects the health of their ecosystems (Burek et al 2008), detecting disturbances in marine environments (Aguirre and Tabor 2004; Bossart 2011b). Studying marine mammals during long time frames may contribute to the evaluation of the current well-being of the ecosystems by allowing the identification of damaging environmental trends, which may reflect on wider aquatic environments and on human health, particularly considering the increasing concentration of human activities in coastal environments (Bossart 2007).

The overall health of an individual animal results from a complex interaction between its immune status, body condition, pathogen characteristics, toxicant load and environmental parameters (Burek et al. 2008). To evaluate marine mammals' health status, baseline data from which change can be assessed needs to be established. Induction of leucocyte cytokine transcription takes place in innate and adaptive immunity and in general inflammatory

responses. Therefore, determining the expression of cytokine genes may be useful for cetacean health assessment due to their broad range of functions and their association with disease in humans and animals (Chaussabel 2015).

Common dolphins (*Delphinus delphis*) are the most abundant small cetacean species in coastal waters of Continental Portugal with an annual average abundance of 45179 individuals (CV=0,25) recently estimated for the period 2010-2015 (Vingada & Eira 2017). They are frequently found stranded along the Portuguese coastline, particularly in the northern half of the coast, where an annual average of 150 dead common dolphins is registered by the regional stranding network each year (Ferreira et al. 2016). As such, due to their high abundance and readily available samples, common dolphins are good candidates for monitoring marine mammal overall health.

Our previous surveys described important pathogens in common dolphins from the Portuguese coast: morbillivirus, herpesvirus and *Toxoplasma gondii* (Bento et al., 2016, 2019). These pathogens, especially morbilliviruses, can modulate the immune system, rendering animals susceptible to other infections. In fact, morbilliviruses are particularly known for their immunosuppressive activity (Griffin 2010; de Vries and de Swart 2014).

Cytokines are messengers of the immune system that are responsible for the induction and regulation of both innate and adaptive immune responses and have distinct functions including the activation of pro-inflammatory and anti-inflammatory pathways (Kennedy 2010; Fenner's Vet. Virol. 2011). IL-6, IL-1β and TNF-α are pro-inflammatory cytokines strongly involved in the innate immune response, potentiating nonspecific pathways such as acute phase response or fever (Cerón, et al. 2005; Tizard 2009). IL-12 and IFN-γ are involved in the acquired immune response and are strongly related to the cellular immune response (Th1 subset activation) (Locksley and Scott 1991; Tizard 2009) while the humoral antibody response (Th2 subset) is associated with IL-4 and IL-10 production (Moss et al. 2002; Moss et al. 2004; Delves et al. 2017). Although most cytokines yield more than one effect, and are often redundant, each part of the immune system has a different and characteristic cytokine pattern (Delves et al. 2017). Recognizing basal levels for cytokine expression will allow detecting shifts associated with disease.

In the present study, cytokine profiling was used to assess the dynamic of the immune response in common dolphins (*Delphinus delphis*) infected with different pathogens. Our study was focused on morbillivirus infection due to its immunosuppressive nature (Moss et al. 2004; Avota et al. 2010; Griffin 2010). The main objective of this study was to evaluate the relative expression of several cytokine genes, targeting interleukin (IL) 1β, IL6, Tissue Necrosis Factor α (TNFα), IL12, interferon γ (IFNγ), IL4 and IL10 in infected and non-infected common dolphins.

2. Materials and methods

2.1. Sampling

Stranded cetaceans are routinely collected and sampled by technicians of the Portuguese National Stranding Network accredited by the Institute for Nature Conservation and Forests. All procedures follow standard protocols (Kuiken and Hartmann 1991) and samples are kept in the Marine Animal Tissue Bank at ECOMARE. For this particular study, all samples were collected by the Northern section of the stranding network. Common dolphin pulmonary lymph nodes (about 200mg) were taken using sterilized instruments and they were preserved in RNAlater and frozen at -20ºC. Also, several tissue samples were preserved in 10% buffered formalin for posterior histology analysis.

In order to establish baseline levels for the targeted cytokines, a group of common dolphins (n=29) presenting no signs of disease were selected from the tissue bank. Following a full necropsy procedure, the determined cause of stranding for these individuals was fisheries bycatch. All animals had signs of recent feeding (presenting intact or partially digested prey in the stomach) and a good body condition (levels 1 and 2 out of a 1 to 5 score) (Geraci and Lounsbury 1993). Also, all 29 animals tested negative for herpesvirus and dolphin morbillivirus (DMV) (Bento et al., 2019; Bento et al., 2016). On the other hand, cytokine expression was also determined in another two common dolphins that tested positive for DMV (Bento et al. 2016). Both animals showed clear signs of disease and one of the DMV positive individuals was also positive for herpesvirus (Bento et al., 2019).

2.2. Histology

To establish the disease status of the two DMV positive individuals, tissue samples were subjected to standard histology procedures, in addition to immunohistochemistry to detect *Toxoplasma gondii* and CeMV. Available tissue samples from both animals included lung, pulmonary mesenteric and pre-scapular lymph nodes, thyroid gland, skin and subcutaneous tissue, adrenal glands, liver, kidney, spleen, urinary bladder, among others. Central nervous system tissue was also processed for histology in both animals. Initially, tissue samples were routinely processed for histology, embedded in paraffin and cut into 4 µm sections. They were stained with hematoxylin and eosin (H&E) in an automated stainer for light microscopy analysis.

Immunohistochemistry (IHC) staining for detection of *Toxoplasma gondii* and morbillivirus antigen was performed using the DAKO PT-link system and the Autostainer-EnVision (DAKO). Positive and negative controls were used for each sample. The primary antibody against *Toxoplasma gondii* was a rabbit polyclonal antibody at a 1:4000 dilution (courtesy of Dr. Bjerkas) and the antibody against Cetacean morbillivirus (CeMV) was a mouse monoclonal commercial antibody (VMRD) against the nucleoprotein of the Canine Distemper Virus that cross reacts with CeMV, used at 1:50000 with an incubation step of 40 minutes. After the washing steps, the secondary antibody was incubated (DAKO Rabbit/Mouse EnVision Detection System K5007) for 40 minutes at room temperature, with the dilution recommended by the manufacturer. Slides were incubated for 5 minutes in DAB-Chromogenhydrogen peroxide (Dako k3468) and stained with Mayer hematoxylin

2.3. Primer design

Specific primers were designed for the amplification of mRNA for IFN-γ, TNFα, IL12, IL-4, IL-10, IL6 and IL1β (Table 9). Primers were also designed for the detection of the housekeeping/reference gene RPL7. Primers were designed (using NCBI Primer Blast tool, Primer3 and Blast) so that at least one in each pair spanned an exon-exon junction, thus ensuring that we favored mRNA rather than genomic DNA. Primers were then purchased from a commercial manufacturer (STAB Vida, Portugal). Relative expression was quantified for each cytokine using Miner software (http://www.miner.ewindup.info), following the computed algorithm for Quantitative Real-time system (Zhao et al., 1995).

Table 9 – Primers used to amplify the targeted cytokines by qPCR and accession numbers for the available sequences (http://www.ncbi.nlm.nih.gov/) used to design them.

Primer	Sequence	Accession numbers
IL4 FOR	5′ GGC ATG TAC CAG CAA CTT CG 3′	XM_007188557 XM_007461337 XM_004279901 XM_007106263
IL4 REV	5′ TGC TGT CAG GAT GTT CAG CG 3′	
IL6 FOR	5′ GCA TCG AGG CTG TGC AGA TT 3′	AF076643 XM_004263443 XM_004330286
IL6 REV	5′ GTT GGG TCA GGG GTG GTT AC 3′	
IL10 FOR	5′ GCC CTG TGA AAA CAA GAG CA 3′	AB775207 XM_004312229 XM_004282421 XM_007470712 XM_007171565 XM_007100340 U93260.1
IL10 REV	5′ ATG GCT TTG TAG ACA CCC TT 3′	
IL12 FOR	5′ TTC CAG TGC CTC AAC CAC TC 3′	XM_007454130 XM_007110551 XM_007187073 XM_004281851 XM_004324402
IL12 REV	5′ CCT CCA CTG TGC TGG TTC TA 3′	
TNFα FOR	5′ GGG AAG AGT TCC CAA CTG GC 3′	XM_004286610 GQ141103 DQ340436 AF320323 NM_001280615 AB049358 XM_007459580 XM_007193976 XM_007110157
TNFα REV	5′ GCA TAT GTG TTC AGC CAC CG 3′	
IFNγ FOR	5′ CTC CTG CAT CAG ACA GGC TT 3′	HQ585526 HQ585529 HQ585525 HQ585527 HQ585528 HQ585522
IFNγ REV	5′ ACC TTC CAG CTC TTC AGC AC 3′	
IL1β FOR	5′ CCC ACC AAC GAA GTG ATG GC 3′	AF320322.1
IL1β REV	5′ GTG GGA GAT TTG CAG GTG GA 3′	
RPL7 FOR	5′ TGC TGT GCC AGA AAC CCT TA 3′	XM_007453712
RPL7 REV	5′ TCC TTG CCT TTC GAA GCA TCT 3′	

2.4 RNA extraction, cDNA and qPCR

A commercial kit by Qiagen (RNeasy) was used to extract nucleic acids. Total RNA was extracted from pulmonary lymph nodes and cDNA was synthesized using a Transcriptor kit (Roche) and used to perform quantitative PCR (qPCR). qPCR was performed in an Applied Biosystems StepOnePlus™ Thermocycler with 50 ng of cDNA, 400 nM of each primer for a final volume of 12,5 µl, with 2x Luminaris Color Higreen High Rox qPCR mix (Thermo scientific). Cycling conditions included 10 min/90°C followed by 45 cycles of 15 sec/95°C and 1 min/60°C and a dissociation step. Amplification curves and melting temperatures were analyzed for each sample. Results were analyzed using Miner (Zhao & Fernald, 2005). This method uses calculations based on the kinetics of individual PCR reactions without the need of the standard curve, allowing direct determination of efficiency and Ct value. Also, this algorithm provides an objective and noise-resistant method for quantification of qPCR results that is independent of the specific equipment used to perform PCR reactions (Zhao & Fernald, 2005). All obtained values were calibrated for the reference gene RPL7.

2.5Statistical analysis

The obtained data did not present a normal distribution and therefore several transformations were analyzed for each cytokine: log; loginv and sqrt (see Results section and Supplemental file 6). Normality was checked using the Shapiro Wilk's test. Results obtained for each transformation were backtransformed for reporting (e.g. for the log transformation, mean values were antilogged to produce geometric means). However, standard deviations and standard errors were not backtransformed; instead, confidence intervals were calculated on the log scale and the confidence limits were antilogged to produce the confidence interval on the original scale. A GLM approach was applied using the Least Square (LS) means of the results from the healthy and diseased individuals, given the imbalanced group sizes. The statistical analysis was performed using SAS software (SAS 9.3; SAS Institute Inc., NC, USA).

3. Results

Histopathology analyses confirmed the disease status of the individuals in the sick group (Dd-302/2012 and Dd-191/2013) (Figure 13). Both animals presented lymphoid

depletion in all analyzed lymph nodes with amyloid deposition, ranging from moderate to severe. Furthermore, both animals presented an active toxoplasmosis: animal Dd-191/2013 had a mononuclear multifocal encephalitis, necrotizing lymphadenitis and lymphoplasmacytic adrenalitis and hepatitis with signs of cirrhosis all associated with *Toxoplasma gondii*; Animal Dd-302/2012 presented lymphoplasmacytic adrenalitis and hepatitis associated with *Toxoplasma gondii.*

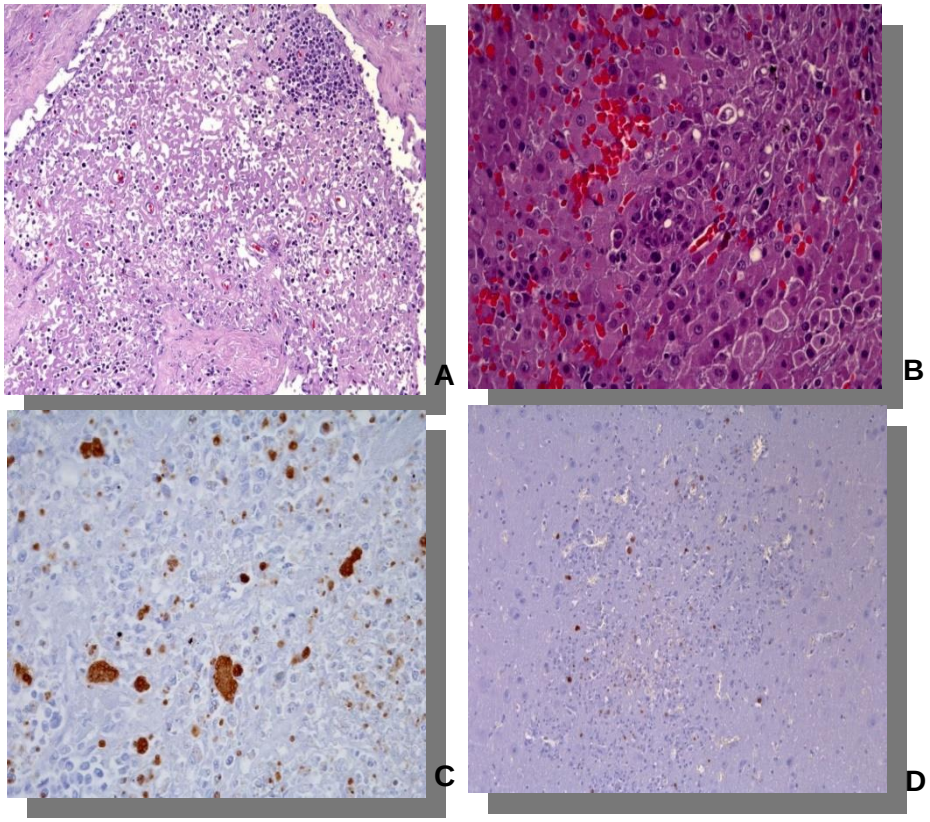


Figure 13 – Histology of lymphnode (A), liver (B), adrenal gland (C) and brain (D) of positive DMV samples. Individual Dd-302/2012: A, (40x) lymphoid depletion with amyloid deposition; B, (100x) liver with multifocal necrosis associated with mononuclear inflammatory infiltrates distributed in the parenchyma, more intense in the periportal area; Individual Dd-191/2013: C, (100x) Toxoplasma gondii IHC of the adrenal gland with numerous bradyzoite cysts; D, (40x) Toxoplasma gondii in the IHC of the brain.

Regarding cytokine expression, in the control group (n=29) IFN, TNF and IL1β presented higher relative expression values, whereas lower relative expression values were

registered for cytokines IL4, IL6, IL10 and IL12. In the sick animals' group (n=2), the expression of most cytokine genes appeared to be downregulated, except for IL10 and IL12 (Figure 14).

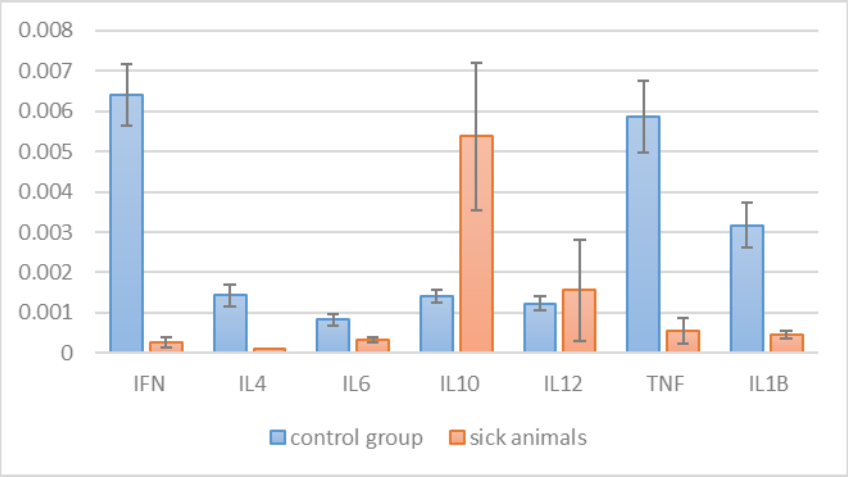


Figure 14 - Mean values (±SE) for cytokine relative expression levels in the control and sick animal groups, normalized for the RPL7 reference gene.

Once dataset normality was met, the proc GLM was used in SAS. Least squares means (LS-means) were calculated for each cytokine considering the effect of the treatment "Healthy" (Y/N). For reporting purposes, results were backtransformed (antilogged or squared, as appropriate) so to present the geometric means for each cytokine. Values for confidence intervals were calculated on the transformed scale and confidence limits were backtransformed to produce the confidence interval on the original scale. Differences obtained between the control group and the sick animal group were statistically significant for IFN, IL-4, IL-10, TNF, IL1β but not for IL6 and IL12 (table 10). The relative expression of IFN, IL-4, TNF and IL1B was significantly higher in the control group than in the sick group, while IL-10 values were significantly overexpressed in the sick group when compared to the control group.

Table 10 - Least square means for the control (Y) and sick groups (N); values shown are backtransfomed mean values and corresponding confidence intervals (CI), and P-values for comparison of treatment effects. Significant values in bold.

	LS-mean CI (min-max)		P-value
	N	Y	
IFN	0.000246 (0.000551 - 0.00301)	0.005661 (0.004219 - 0.007314)	**0.0054**
IL4	0.000104 (1.33E-05 - 0.00081)	0.000939 (0.000632 - 0.001395)	**0.0399**
IL6	0.000312 (0.000132 - 0.00074)	0.000676 (0.000527 - 0.000867)	0.0886
IL10	0.005064 (0.001998 - 0.012835)	0.001166 (0.000901 - 0.001509)	**0.0043**
IL12	0.001241 (0.00028 - 0.002886)	0.001099 (0.000774 - 0.001482)	0.8263
TNF	0.00046 (0.000138 - 0.001539)	0.004334 (0.003101 - 0.006058)	**0.0011**
IL1β	0.000439 (0.000123 -0.00157)	0.002228 (0.001554 - 0.003194)	**0.0182**

4. Discussion

In the present study, we established baseline mean cytokine expression values (with low associated SE) in a group composed of 29 common dolphins stranded along the Portuguese coast. These baseline values refer to a control group and represent a valuable tool to evaluate the health status of stranded cetaceans. The comparison of this baseline cytokine profile with the cytokine profile of individuals infected with cetacean morbillivirus (Bento et al. 2016) and with active toxoplasmosis, allowed the evaluation of the immunological shift (a downregulation of the expression of IFNγ, IL-4, TNFα and IL1β and an upregulation of the expression of IL10) promoted by these pathogens.

In general, all morbilliviruses are known to have a high lymphotropism and their affinity to the SLAM protein plays a major role in lymphocyte infection and immunosuppression, as previously described in other species (Messling et al. 2006). Severe leukopenia usually ensues due to apoptosis in cells of the immune system and, even after viral elimination from peripheral blood, a decrease in antigen presentation and lymphocyte maturation is thought to contribute to the continuation of the immunosuppressant status in morbillivirus infections (Beineke et al. 2000).

After the acute phase of morbillivirus infection, a shift in cytokine production from type I to type II T cell cytokines is observed in humans with morbillivirus infection (Griffin 2016).

Our results show a decrease in the expression of innate immune response (such as IL1β and TNFα) and of the Th1 subset (Il-12 and IFNγ), and an increased expression of IL-10, from the Th2 subset, that seemingly confirms this immunologic shift.

In cetaceans there is few information available on «in situ» cytokine expression. However, a recent study by Díaz-Delgado et al. in 2019 analyzed local inflammatory responses in lymph nodes from CeMV-infected cetaceans, as had been previously done in infection by terrestrial morbilliviruses. In this study, increased expression of CAS3 in mononuclear cells was found in lymph nodes of CeMV-infected cetaceans. Cas3 plays a major role on cellular apoptosis by catalyzing the specific cleavage of many key cellular proteins (Porter and Jänicke 1999). Furthermore, studies in humans have implicated IL-10 in cellular apoptosis, including that of Th1 cells (Moore et al 2001). Our samples from CeMV-infected cetaceans have shown varying degrees of lymphoid depletion with amyloid deposition with an overexpression of IL-10. In the future, it would be of interest to assess CAS3 expression in these samples.

The role of IL-10 in immunosuppression is paramount. IL-10 is an anti-inflammatory cytokine known to inhibit several immune responses, namely the production of pro-inflammatory cytokines such as TNFα (Fiorentino et al. 1989). Continuous IL-10 production induces suppression of cytokines produced by Th1 cells and decreases T cell proliferation leading to immunosuppression as observed in the late phase of generalized bacterial infections (Song et al. 1999). Furthermore, overexpression of IL-10 induces apoptosis of CD4+T cells, as demonstrated in septicemic mice and humans (Akdis and Blaser 2001). Considering marine mammals, an inhibitory effect of IL-10 on peripheral T lymphocytes leading to splenic depletion was already hypothesized in porpoises (Beineke et al 2007).

Both sick animals presented an active toxoplasmosis with positive immunostaining in several organs, with signs of inflammation and necrosis. A study by Neyer et al (1997) revealed that IL-10 plays an important role on the susceptibility of mice to Toxoplasma gondii and it could be linked with both protection or increased susceptibility to disease. In fact, in mice lacking lymphocytes (SCID mice), endogenous IL-10 was associated with increased susceptibility to T. gondii (Neyer et al. 1997). This increased susceptibility to toxoplasmosis in immunocompromised animals with high IL-10 levels could help explain why toxoplasmosis in free roaming cetaceans has been frequently detected in association with CeMV infection (Domingo et al. 1992; Bressem et al. 2009; Mazzariol et al. 2012; Profeta et al. 2015; Bento et al. 2019b).

The availability of samples from free roaming cetaceans is a limitation towards establishing normal range values for biological parameters. However, high rates of incidental captures by fisheries worldwide provide large numbers of bycaught cetaceans (including Portugal, see Vingada & Eira 2017). These individuals die because of an acute trauma and

are frequently otherwise healthy with good body condition, as confirmed at necropsy. As a result, sampling cetaceans from bycatch events is a good option when trying to establish baseline values for healthy cetacean populations.

Results obtained in this work are preliminary since they originated from only two “non-healthy” common dolphins. A larger sample size will be needed to further corroborate the observed changes in common dolphins with morbillivirus infection. Nonetheless, our results indicate that IL-10 was the only cytokine that was upregulated in the lymph nodes of animals with histological signs of immunosuppression and positive to morbillivirus infection. Considering its role in immunosuppression and its ability to downregulate other mechanisms of the immune system, our results support a generalized and marked immunosuppression that was maintained over a considerable period of time after infection with morbillivirus, allowing for the establishment of an active toxoplasmosis.

This work was partly funded by the Portuguese Wildlife Society and projects SafeSea EEA-Grants, European Commission's Life Programme (project LIFE+MarPro NAT/PT/00038) and project FCT CetSenti RECI/AAG-GLO/0470/2012 (FCOMP-01-0124-FEDER-027472), FCT/MCTES (PIDDAC) and FEDER–COMPETE (POFC). Thanks are due to Foundation of Science and Technology/Ministério da Ciência, Tecnologia e Ensino Superior (FCT/MCTES) for the financial support to CESAM (UIDP/50017/2020+UIDB/50017/2020 + LA/P/0094/2020) and to CE, and Grants SFRH/BD/30240/2006 to MF.

CHAPTER V

Discussion on specific case reports

Introduction

Molecular surveys are paramount to understand the epidemiology of viral infectious diseases by allowing tracking disease spread and genetic evolution of viruses. Furthemore, surveying stranded animals gives us a window of opportunity to monitor the health status of these animals and to pinpoint possible threats to these populations. Despite this, it is also important to look at these animals from an individual standpoint and pay closer attention to the effects of viral infections on affected individuals. Although many of the animals that test positive for morbillivirus were live strandings, we lack clinical information on these animals since they died shortly after the stranding event, preventing clinical examination and diagnostic tests while they were still alive.

Collecting tissue samples during necropsies for different purposes allows not only studies on prevalence of disease but also allows the retrospective histopathological analysis of infected animals to infer course of infection, cause of death and to detect comorbidities.

The next case reports allow encompassing all the available information on these individuals to further characterize their health status and eventually the cause of death. Information gathered for each of these animals is summarized in the next case reports.

Case 1

In this animal, CeMV was detected by qPCR only in the brain. Histologically, this individual had a moderate lymphoid depletion in the lymph nodes and a lymphoid follicular hyperplasia in the spleen. A mononuclear encephalitis was detected and positive staining for DMV was detected in the IHC analysis of brain tissue, with positive groups of neurons present in the areas of parenchymal inflammation (image 15). Vacuolization of the white matter was seen in this animal, similarly to observations from other authors on animals with morbillivirus infection secondary to edema (Duignan et al 1992; Klemens et al. 2019). No other organs tested positive for DMV in the IHC analysis or presented any signs compatible with CeMV infection.

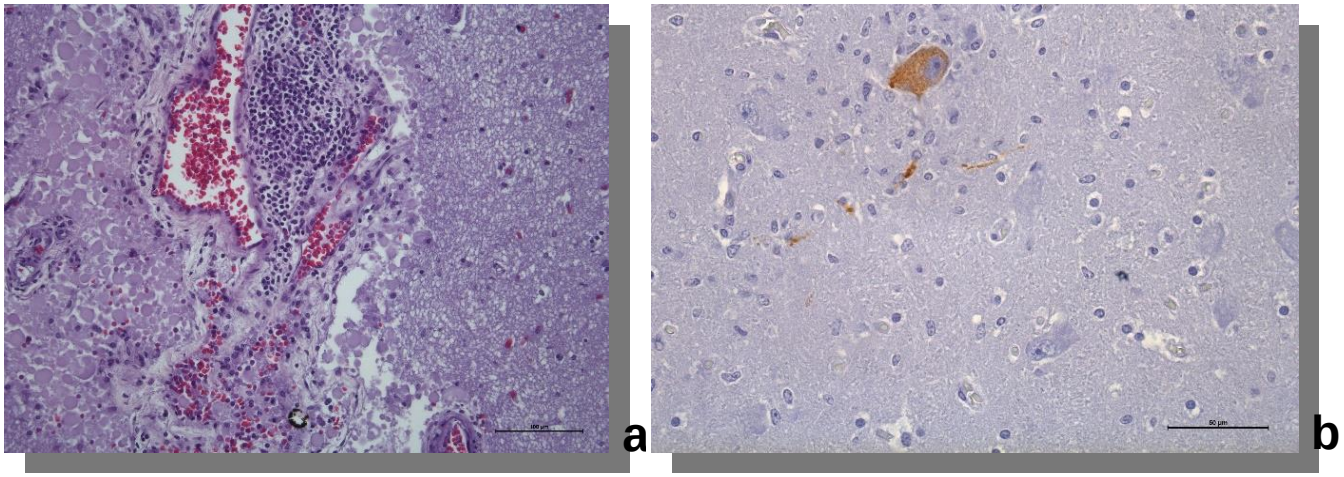


Figure 15 – Central nervous system of a striped dolphin with DMV infection. a – Moderate lymphoplasmacytic perivascular cuffing and white matter vacuolization on the cerebral cortex; b – immunohistochemistry of the brain with positive staining for DMV.

Case 2

In one striped dolphin, CeMV amplification occurred in all tested organs: lung, brain, lymph nodes, kidney, spleen and liver. This animal was also positive for an alpha-herpesvirus in the lung, diaphragmatic lymph node and kidney. Microscopically, it presented a purulent-catarrhal broncho-pneumonia with nuclear viral inclusions; lymph nodes showed lymphoid depletion and syncytia formation with basophilic intranuclear inclusions; periportal intersticial mononuclear hepatitis and a light, focal mononuclear encephalitis. However, the IHC analysis for CeMV was negative in all tested organs probably indicating a low viral load, considering the lower sensitivity of IHC when compared with molecular diagnostic tools. The animal's lesions were compatible with a systemic herpesviral infection, particularly considering the morphology of the viral inclusions, which was identified as the probable cause of death.

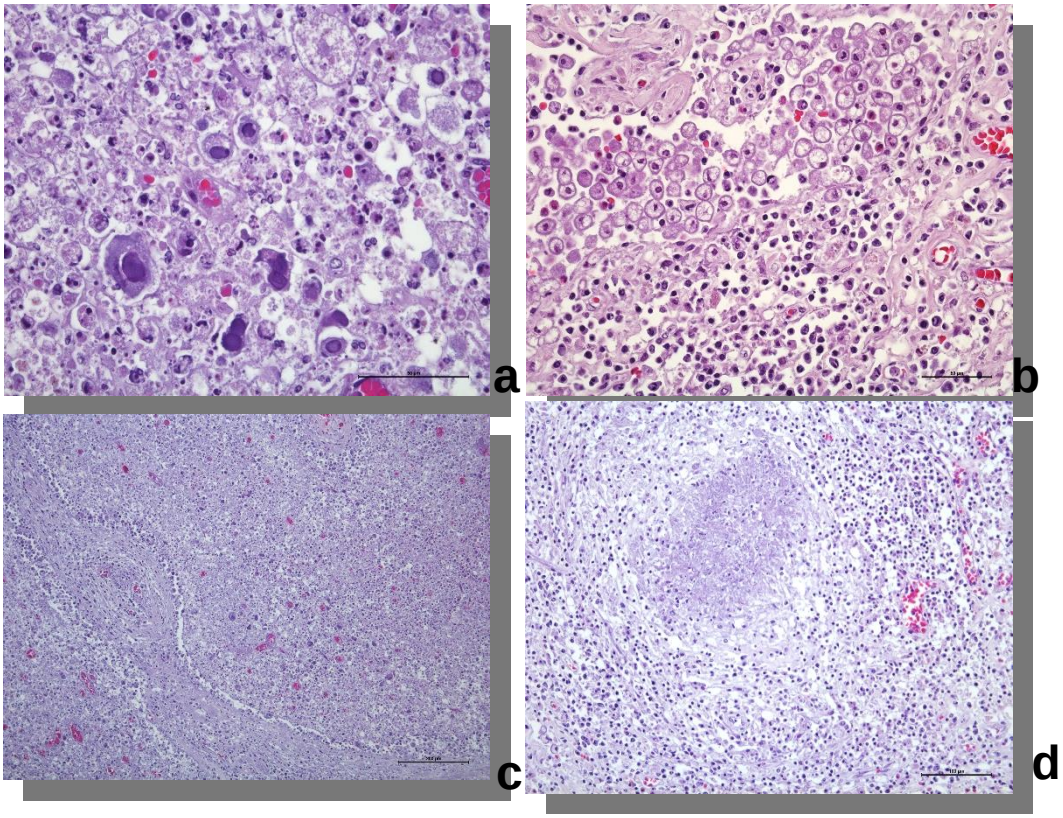


Figure 16 – Histology of the lymph nodes of a striped dolphin positive to DMV and HV. a – diafragmatic lymphnode with marked lymphocitolisis with intranuclear basophylic inclusion bodies; b – mesenteric lymphnode with lymphoid depletion and abundant hystiocytes in the subcapsular sinus. Multifocal foci of necrosis in the follicular areas; c – general view of lymphoid depletion on the diafragmatic lymphnode; d – mesenteric lymphnode with necrosis in the follicle area.

Cases 3 and 4

Two common dolphins also presented chronic systemic CeMV infection. In one individual, CeMV antigen was detected by qPCR in the lung, diaphragmatic lymph node and mesenteric lymph node even though the organs tested negative for CeMV (lung, diaphragmatic LN, thyroids, brain) in the IHC analysis. This animal showed a generalized lymphoid depletion with amyloid deposition in the lymph nodes. IHC and histopathology revealed an active toxoplasmosis associated to a necrotizing hemorrhagic adrenalitis and lymphoplasmocitic necrotizing hepatitis, with bradyzoit cysts in the liver and tachyzoites in

the adrenal glands. This animal probably survived the early stages of morbillivirus infection only to succumb to an opportunistic infection by *Toxoplasma gondii* after a long period of immunosuppression, with a marked lymphoid depletion still present.

With respect to the other common dolphin showing chronic systemic CeMV infection, CeMV was detected by qPCR in samples of lung, brain, liver, kidney, spleen and lymph nodes. Microscopically, this animal was also negative in the IHC analysis for CeMV and also had an active toxoplasmosis. It had a mononuclear multifocal encephalitis, a necrotizing lymphadenitis, a mononuclear necrotizing adrenalitis, a lymphoplasmocelular hepatitis and hepatic cirrhosis, all associated with *Toxoplasma gondii*. In this case, lymph nodes were also severely depleted and with amyloid deposition.

Case 5

Another common dolphin (live stranding) presented a subacute broncho-intersticial pneumonia as the most significant finding. This animal was positive for herpesvirus in the lung and in the kidneys. No viral inclusions were histologically identified but it is possible that a viral pneumonia was later complicated with a secondary bacterial infection. In this animal, two different sequences were amplified, showing a co-infection with different alpha-herpesvirus. The pneumonia was not ruled as the cause of death but raises the possibility of these alpha-herpesvirus being capable of causing severe and potentially fatal disease, as hypothesized previously by Sierra et al., (2014).

Discussion

Except for one striped dolphin, all the animals positive for DMV on the qPCR were negative in the IHC. It is possible that low viral loads are responsible for this situation, since qPCR is generally more sensitive than IHC. This is corroborated by a previous study, in which 86% of the samples negative for DMV in the IHC testing were later found positive by molecular techniques (Taubenberger et al. 1996).

Although in case 1 the animal had probably a higher viral load than other samples tested for IHC and showed vacuolization of the white matter that suggests the presence of some degree of edema, it had most likely a form of chronic localized encephalitis. The

lymphoid depletion and the mononuclear infiltrates in the brain are usually seen in a more chronic course of infection.

A recent study identified a regeneration of lymphoid tissues in chronically infected cetaceans, with follicular, para-cortical and medullary cord expansion (reactive hyperplasia), similarly to what is described for other species (Díaz-Delgado et al. 2019). As already mentioned, in the striped dolphin here reported there was a follicular hyperplasia of the spleen despite the lymphoid depletion in lymph nodes. The cellular proliferation of lymphoid tissues does not necessarily imply a recovery of the immune response to other antigens. Studies performed with MeV showed that by infecting and eliminating preexisting memory cells that express high levels of CD150, this virus erases the memory of past exposures to infectious agents and sets the host's defense system back to its default (Haeryfar 2020). This is referred to as "immune amnesia". The rapid oligoclonal expansion of MV-specific lymphocytes and bystander cells masks this depletion, explaining the short duration of measles lymphopenia yet long duration of immune suppression (de Vries et al. 2012). Two other striped dolphins that tested positive for CeMV by qPCR in the brain also did not show any signs of lymphoid depletion and it would be interesting to assess the immune response in these animals.

Histology performed in animals with chronic systemic infection by CeMV revealed a marked lymphoid depletion, as previously described by Stephens et al. (2014) and on those animals no signs of lymphoid hyperplasia was detected.

It is also interesting to notice that both common dolphins with DMV showed active toxoplasmosis, further emphasizing the immunosuppressive nature of this virus.

Molecular surveys associated with detailed necropsy observations and histology provide a valuable insight into the cause of death of these animals and provides information on the pathophysiology of these diseases.

CHAPTER VI

General discussion, conclusion and future perspectives

1. General discussion

Over the years, a great deal of scientific knowledge on cetacean species has been obtained from stranded individuals. However, the information drawn from sampling stranded cetaceans has to be carefully assessed and its use is frequently a cause of debate. Studying free roaming cetacean populations can be challenging, mainly due to the lack of access to biological samples. Collection of samples from live free roaming specimens poses several concerns. Remote biopsy sampling techniques are described and were used, for instance in the US as a part of the Natural Resource Damage Assessment to evaluate impacts from the *Deepwater Horizon* oil spill on bottlenose dolphins and other marine mammals off the northern Gulf of Mexico (Sinclair et al. 2015). These techniques only allow the collection of skin and blubber limiting the studies that may be performed. Although this technique is described as fast and causing little disturbance, it is not risk free. In 2000, a report of a direct death of a common dolphin in the Mediterranean Sea caused by a remote sampling device was published (Bearzi 2000). Apart from remote biopsies, other samples can be obtained from free roaming cetaceans. In large mysticetes, collection of fecal samples and blowhole droplets is possible, although logistically demanding (Hunt et al. 2013). These sampling techniques not only raise ethical concerns over the invasiveness of the process and on the limitation of samples but also over the costs of operation in order to obtain a significant amount of samples. On the other hand, stranded animals allow the collection of a vast array of samples and the systematic gathering of data from stranded animals is of extreme importance for the determination of cause of death. In Portugal, bycatch is very frequently identified as cause of death for stranded cetaceans (Vingada & Eira 2017) and therefore gathering information from stranding events is paramount for the establishment of mitigation measures for cetacean interactions with fisheries.

Stranding events are common worldwide and they can occur with single individuals, groups or even in the form of unusual mortality events (UMEs). UMEs are considered unexpected, involving a significant die-off of marine animals and demand an immediate response (Wilkinson 1996). These UMEs can occur in the form of stranding events, but not always. In Portugal, beach seines are a traditional artisanal fishery used mainly in the summer in the central region of the country. This technique is responsible, from time to time, for the bycatch of groups of marine mammals, mainly common dolphins and sometimes porpoises. Besides the seasonal mortality associated with beach seines, mortality of cetaceans is seen year-round, with a high percentage of stranded individuals (up to 54%) being confirmed as resulting from fisheries bycatch (MarPro and Pr 2012).

The 1994 amendments to the 1972 US Marine Mammal Protection Act (MMPA) created the potential biological removal (PBR) approach to determine the level of human-caused mortality marine mammal populations could sustain while still allowing those populations to recover (Wade 1998). In Portugal, mortality estimates for several species were calculated in previous studies using abundance estimates for a 5 year period (2010-2015) and specific mortality rates for the geographic area studied in order to determine the PBR value for several species, pondering the rates of carcass recovery for each species (Vingada & Eira 2017). Results showed that PBR rates are high for all studied species except for striped dolphins, probably because being an oceanic species, they are less prone to strand on the coast and/or because they are more susceptible to bycatch in different types of fishing gear. The porpoise PBR (8.4%) was much higher than the commonly accepted threshold of 1.7% of the population. Porpoises are mainly coastal animals and use areas where small-scale fisheries are particularly active. The overlap between fishing areas and porpoise habitat is suspected to be one of the reasons for the high rate of porpoises stranded due to bycatch in Portugal. This is particularly worrisome if we consider the proposed Iberian subspecies (*Phocoena phocoena meridionalis*) (Fontaine et al. 2014) since this could mean they may face rapid decline over the next years. Also, although common dolphins are, by far, the most abundant species in our coast, the high number of stranding events each year is reflected in a relatively high PBR (7.52 %) that raises long term concerns towards this population (Vingada & Eira 2017).

In the present study, common dolphins are the most tested species because they represent the largest group of samples available due to the high numbers of stranding events. Although porpoises were not as frequent, they were also included in the viral surveys that were performed. The results for this species were particularly important because of their vulnerable conservation status in comparison to the other studied species. On the other hand, striped dolphins are a particularly interesting species to study because of the morbillivirus infection events reported in the past (P J Duignan et al. 1992; Edwige Nina Bellière et al. 2011). However, since striped dolphins are mostly oceanic rather than coastal they are not frequently found stranded in the Portuguese continental coast when compared to other more coastal species (Vingada & Eira 2017).

In this work we surveyed 279 animals for the DMV strain of CeMV, including 33 animals from Galicia, Spain, and the remaining from Portugal. Our results indicate a higher prevalence of DMV among stranded striped dolphins (20.6 %) when compared to stranded common dolphins (1 %) from the Atlantic based populations and no positive samples were detected among the other species tested.

Positive samples for DMV genome were detected annually in the period of 2007-2013, showing that the virus is circulating in cetacean populations from the Atlantic off the

coast of Portugal and northern Spain and both striped dolphins and common dolphins were found to be positive to viral infection.

The percentage of positive animals (24.2%) from Galicia was probably strongly underestimated, since available samples were limited and mainly consisted of lung samples frozen at -20°C, limiting our chances of detecting CeMV. The difference in percentage between Galician (24.2%) and Portuguese (16.7%) samples was not statistically significant, but the proportion of Galician positive samples could be much higher if sample collection and storage was more appropriate. Therefore, a survey of the Striped dolphin population of the Bay of Biscay would be particularly interesting, where a higher prevalence may be detected.

Within the temporal range of our study (2007-2013), no UMEs involving striped dolphins were detected, and positive animals were detected yearly. Unlike the Mediterranean striped dolphin population, morbillivirus infection appears to be endemic in the tested striped dolphin population of the Atlantic ocean. This correlates with the serological survey conducted in 2011 in which 21.6 % (n = 37) of the analyzed cetaceans cross-reacted with Canine Distemper Virus antigen in a commercially available ELISA kit (Bento 2011).

Unlike striped dolphins, the prevalence of stranded common dolphins positive for viral antigen was much lower (1 %). The difference in CeMV prevalence between stranded common and striped dolphins needs to be fully assessed and further studies are needed to clarify the virus impact on cetacean populations and why do striped dolphins appear to be more susceptible to DMV infection.

SLAM (CD150) is the major cellular receptor for the entry and replication of morbilliviruses in target cells of humans, cows and dogs (Tatsuo et al. 2000; Tatsuo et al. 2001). Binding between SLAM and the H protein of morbilliviruses triggers subsequent cell fusion events via viral fusion protein, allowing viral invasion of host cells (Hashiguchi, Ose, et al. 2011; Hashiguchi, Maenaka, et al. 2011). This binding happens through the V domain of the SLAM receptor for which morbillivirus' H protein has strong affinity (Shimizu et al., 2013). The identification of the SLAM cell receptor in several cetacean species was a major step in our understanding of the pathogenesis of CeMV infection, especially with regard to susceptibility and transmission to non-classical hosts. Substitution experiments on the amino acid residues of the human SLAM interface have shown that some residue substitutions lead to a loss or reduction or sometimes an increase in, viral infectivity, indicating the key role of these residues for both binding affinity and viral infectivity. The same has been hypothesized in marine mammals (Ohishi et al. 2010; Shimizu et al. 2013c). Shimizu et al evaluated the variation pattern of the amino acid residues in the V region of the SLAM receptor of 26 cetacean species, covering almost all cetacean genera; bottlenose and striped dolphins were found to have substitutions at five positions (E68G, I74V, R90H, V126I, and Q130H) compared with those of baleen whales. Three residues (at positions 68, 90 and 130) were

found to alternate electric charges, possibly causing changes in affinity for the virus (Shimizu et al. 2013c). The variation pattern of amino-acidic motifs in the viral receptor SLAM should be further investigated in order to assess the practical significance of these detected changes for the susceptibility of these species.

In the Mediterranean, Striped dolphins have consistently been the most affected by CeMV. Striped dolphins are the most abundant Odontoceti species, living in large pods which could enable an easy spread of the disease (Van Bressem et al., 2014; J. A. Raga et al., 2008; Rubio-Guerri et al., 2013; M. Van Bressem et al., 1991). Also, Mediterranean populations are probably relatively isolated from Atlantic populations (Bourret et al. 2008) and a high degree of inbreeding could justify some genetic susceptibility (Valsecchi et al. 2004). Serological results showed that prior to the second epidemic (2006-2008) antibody titers were low, indicating that apparently the population had again become susceptible to infection (Van Bressem et al. 2001).

With respect to morbillivirus, in the present study the phylogenetic trees for the concatenated nucleotides showed groups of viral sequences following a temporal arrangement, with samples collected since 2007 forming different clades. The trees for concatenated sequences were generated with few sequences since not many sequences were available. By narrowing our analysis to the P gene, the number of available sequences increased substantially. After adding more sequences to the trees (P gene tree), a phylogeographic arrangement became clear: all samples from Portugal and Galicia clustered together (with isolates ranging from 2007 to 2014), farther away from samples from the Mediterranean.

In the present study, only one sequence (from a striped dolphin stranded in 2007) clustered with Mediterranean samples and samples from the Canary Islands, rather than with the other Atlantic samples. This seems to corroborate the hypothesis that these populations may be relatively isolated from each other, and also the possibility of viral introduction from the Atlantic into the naïve Mediterranean population (J.A. Raga et al. 2008).

Regarding HV, we surveyed 179 cetaceans stranded along the Portuguese coastline and found a total percentage of positive animals of 7.8% (14/179). The percentage of HV positive animals increases to 14.3% (10/70) when considering only samples from fresh carcasses and this difference is significant if compared to the percentage of positive samples when decomposed carcasses are included.

Herpesvirus sequences were detected using the previously described system (VanDevantier et al, 1996), which allows the amplification of a conserved region within the DNA polymerase gene shared by the three subfamilies of herpesviruses. Due to the use of highly degenerate primers allowing the amplification of highly diverse nucleotide targets, there is a strong possibility that prevalence was considerably underestimated.

A qPCR assay would be much more efficient, but so far, and due to the few available sequences and the high variability among them we were not able to develop a more efficient detection system. Studies using similar detection methods in other species obtained higher percentages of positive samples: 25.8% in chimpanzees (Seimon et al. 2015), 27% in rodents (Ehlers et al. 2007) and 26% in humans (Minjolle et al. 1999).

The fact that these samples were obtained in stranded animals already in different decomposition degrees, allied to the fact that this pan-herpesvirus system may have a low sensitivity to detect cetacean herpesvirus, as already reported by Vandevantier et al, 1996 for different human herpesvirus, may justify these numbers.

The reported results must be carefully analyzed since their value, as a true survey is very limited. However, information regarding pathogen incidence in free roaming cetacean populations is important, particularly for herpesvirus, which is able to establish a latent lifelong infection, whose reactivation is related to environmental stress. Additionally this kind of molecular survey provides important genomic data, increasing the information concerning herpesvirus sequences allowing the development of new tools for the detection of these viruses. Also, the obtained sequences allow for the subfamily allocation, giving us information on strains that may circulate in these populations.

So far, gammaherpesviruses had only been found in cetaceans associated with skin and genital lesions (Smolarek Benson et al. 2006; Lecis et al. 2014; van Beurden et al. 2015), although in other species they have been found to cause systemic disease, such as the malignant catarrhal fever of ruminants (Russell et al. 2009). In this work, a gammaherpesvirus was detected in animal SC-189/2013, which showed histological signs of a systemic viral infection, compatible with herpesvirus infection. Despite this fact, we must be cautious in attributing the clinical signs to a gammaherpesvirus since it is possible to have co-infections with other herpesviruses, as seen in other animals.

In a previously published paper by Sierra et al., 2014, it was suggested that there could be a pathogenic branch of alpha-herpesviruses in cetaceans that could be responsible for fatal cases worldwide. In our study, one striped dolphin and one common dolphin in which an apha-herpesvirus was detected seem to corroborate this hypothesis, with signs of severe viral disease. Although herpesvirus infection is generally benign, it can cause severe disease, especially if the host has a compromised immune system.

With respect to the herpesvirus phylogenetic analysis, the tree generated from the obtained and previously reported sequences displayed three distinct clusters within the Portuguese alpha-herpesvirus sequences. Alpha-1 and alpha-3 clades did not cluster with any of the previously identified genera within the alpha-herpesvirus subfamily, but the alpha-2 clade is close to varicellovirus genera, with low PP value between branches. It would be interesting to amplify further sequences of these viruses in order to assess if these clusters

could in fact be a part of an already established genera. In previous studies by Maness et al (2011) sequences contained in the alpha-2 subfamily clustered within the varicellovirus genera, although it was noticed in Maness's work that shorter sequences would fall out of the varicellovirus clade (Maness et al. 2011). In the gammaherpesvirus subfamily, no specific clades were observed.

Regarding the cytokine expression, we were able to establish baseline values for the relative expression of several cytokines in a group of common dolphins considered healthy free-roaming individuals. This allowed us to compare those results with those of two common dolphins with morbillivirus. Considering our results, we can observe a downregulation of the expression of IFNγ, IL-4, TNFα and IL1β and an upregulation of the expression of IL10.

Morbillivirus's V protein is the primary viral immune interference protein, targeting the innate host response at multiple levels (Caignard et al 2008; Childs et al., 2006; Ramachandran et al 2008b). A rapid shutdown of the host cell quickly impairs the innate immune response to the infecting virus and hampers the production of critical proteins such as class I MHC antigen and antiviral cytokines. Without effective innate immune responses, the infecting virus can quickly replicate and disseminate before the host can develop an adaptive immune response. This strategy is widely used by RNA viruses, many of which have very rapid replication cycles (Fenner's Vet. Virol, 2011).

A shift in immune response favoring Th2 over Th1 is usually observed in MV infection. In Measles, while the early infection phase is characterized by secretion of TNF and IFNγ, indicative of type-I immune responses, later stages of the infection are characterized by elevated levels of IL-4 and IL-10 that may both partially contribute to immunosuppression (Couper et al 2008; Griffin, 2016; Schneider-Schaulies et al 2009; Yu et al., 2008). Furthermore, studies have shown that the production of IL-10 during infection with Toxoplasma gondii mediates the suppression of lymphocyte proliferation observed during acute toxoplasmosis, as well as susceptibility to infection with this parasite, playing a major role in the establishment of immune suppression. Co-infection with morbillivirus and toxoplasma in the two common dolphins studies in this work may have enhanced the production of il-10 that contributed to the severe immune suppression and consequent death of these animals, showing that toxoplasmosis may be an important pathogen for populations susceptible to DMV infection, worsening the health status of these animals and leading them to death.

2. Conclusion

Stranded animals are a valuable source of samples and information that allows us to build knowledge on these species, through the analysis of biological material that would otherwise be unavailable. Working with this kind of samples poses several difficulties since results cannot be directly transposed to the species population as a whole and sampling may be biased towards particular species, demographic groups or cause of death. Samples from stranded animals provide an opportunity to better understand the challenges these species face in the wild, but they may compromise our view of what happens to those that survive since they constitute a bias and do not necessarily represent the living population, especially if we focus on sick animals. The high percentage of animals bycaught in fishing gear allows for samples that may be more representative of the whole population, since the vast majority of these animals died a violent and traumatic death and were, in many cases, healthy providing a brief glimpse of the actual environmental conditions of the marine ecosystem.

The present study revealed that the identified morbillivirus is endemic among the studied striped dolphin population, contrary to what is apparently the case of common dolphins. We identified severe chronic systemic infection with comorbidities in common dolphins, while in striped dolphins we found not only cases of chronic systemic infection but also of chronic localized encephalitis and also signs of RNA persistency in animals without signs of morbillivirus infection.

To the author's knowledge, this is the first report of herpesvirus infection in common dolphins, as well as co-infection with morbillivirus co-infection with more than one herpesvirus strain in these species. The phylogeny of these samples allocated them to alpha and gammaherpesvirus.

The cytokine expression evaluation of common dolphins with chronic systemic morbillivirus infection revealed a marked up-regulation of IL10 and down-regulation of other cytokines such as IFNγ and TNFα. These animals' immunosuppression was detectable not only by the marked generalized lymphoid depletion but also by an ensuing active toxoplasmosis, highlighting the importance of surveilling ecosystem health and pathogen loads on marine environments since they can have a synergistic negative effect on populations' health.

3. Future perspectives

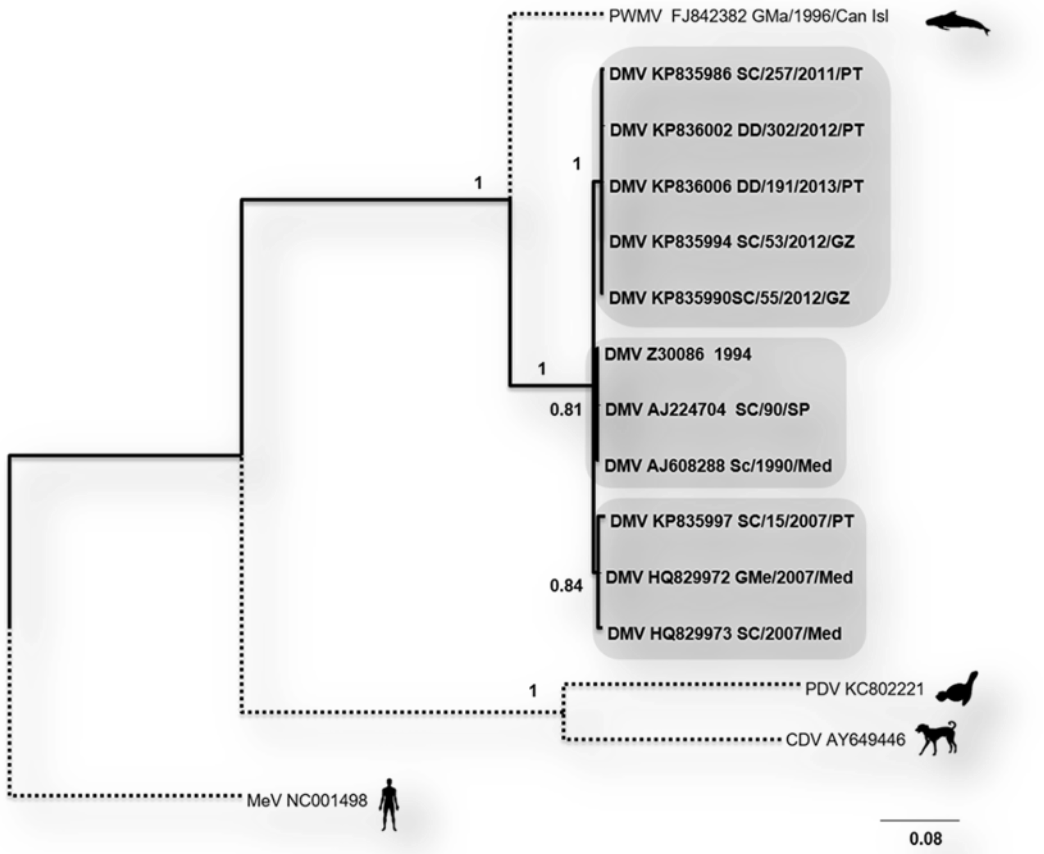
The present study revealed the need for more studies on infectious disease of marine animals, with special enphasys to viral diseases. Although it has been extensively studied in marine animals, morbillivirus infection is still a current issues, especially regarding immunological response differences among cetacean species. The differences on susceptibility and course of infection between animal species raises pertinent questions that still need to be assessed.

Herpesviruses are particularly interesting for their virus-host co-evolution and also for their great variability. The relationship between marine and terrestrial herpesvirus needs to be studied in greater depth especially considering that human activities pose an increased pressure in marine ecosystems and likely has an impact on pathogen loads to which these animals are exposed to, similarly to what happens with *Toxoplasma spp*. Also, sequencing of further HV genomic regions would provide useful information on HV phylogeny in cetaceans.

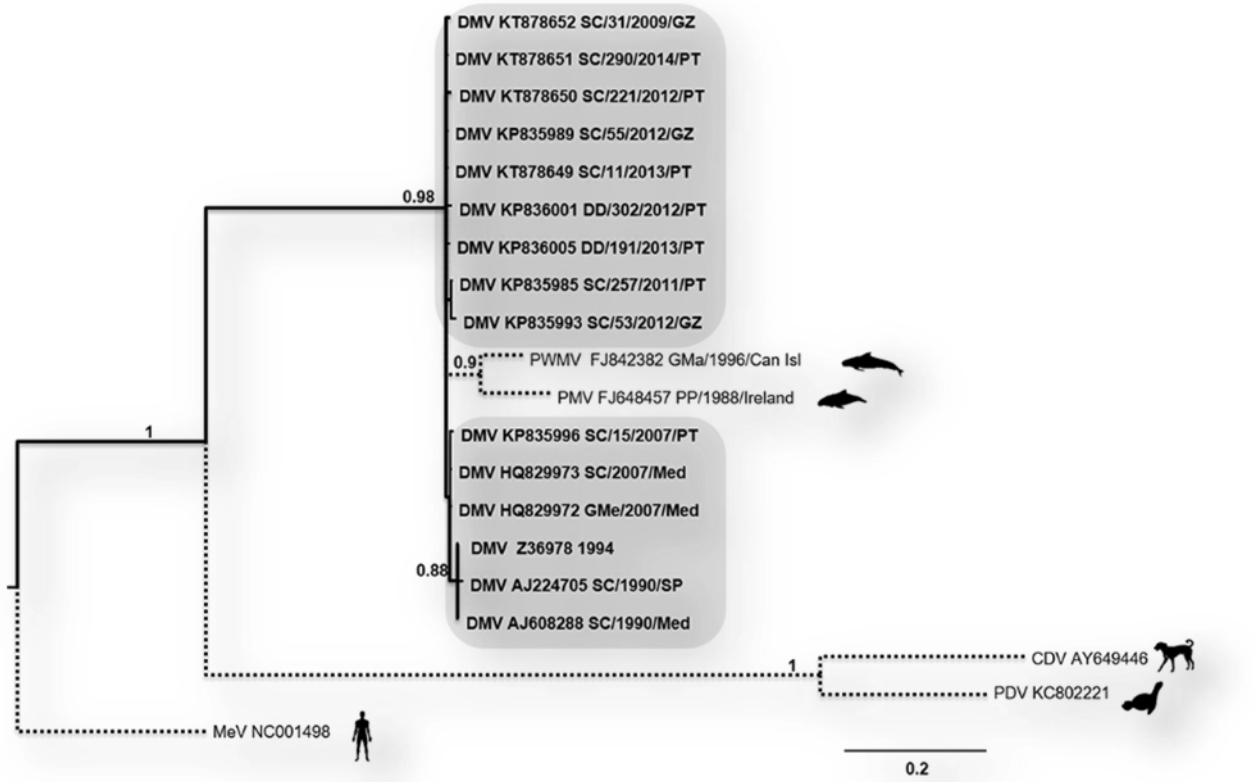
It would also be interesting to assess cytokine levels for other cetacean species, particularly Striped dolphins, which are particularly affected by morbillivirus in the Mediterranean and Eastern Atlantic populations, as well as to assess «*in situ*» cytokine expression.

The importance of marine ecosystems and marine species has been on the rise in part due to the constant threats posed by human actions with consequences such as water pollution, pathogen dissemination and climate change. All these can shape the future of the oceans and contributions to the knowledge on the species that inhabit our seas has now more relevance than ever.

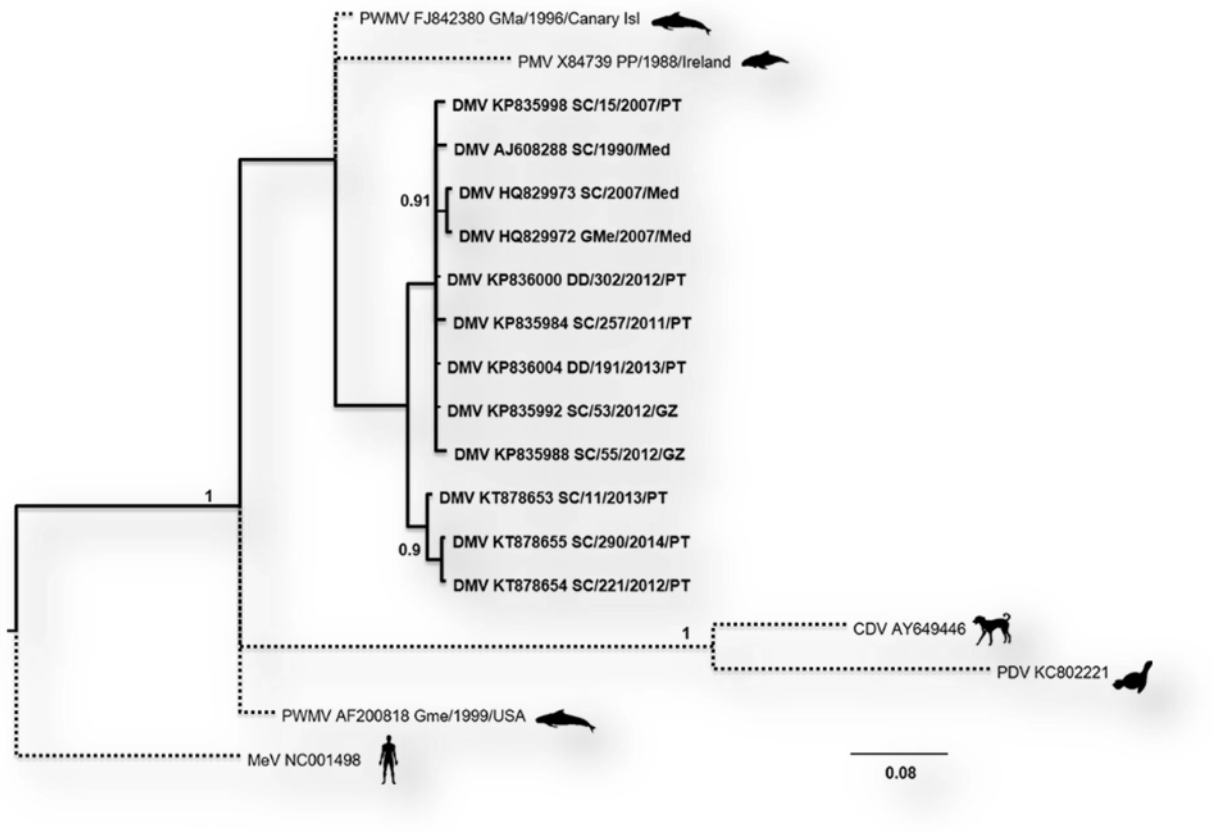
Appendix 1 – Supplementary files



Supplementary file 1: Phylogenetic tree for the F gene nucleotidic sequences. Phylogenetic tree generated with the aligned sequences for the F gene, inferred by Bayesian methods. Sequences for the outgroup taxa were retrieved from NCBI for Pilot Whale Morbillivirus (PWMV [accession number FJ842382]); Phocine Distemper Virus (PDV [accession number KC802221]); Canine Distemper Virus (CDV [accession number AY649446]) and Measles Virus (MV [accession number NC001498]). Three DMV sequences from the 90's were retrieved from NCBI and included in the trees (Z30086; AJ224704 and AJ608288) along with two sequences from 2007 (HQ829972 and HQ829973). Sequences obtained for the F gene of Portuguese and Galician isolates were also included for animals Sc/257/2011 (KP835986), Dd/302/2012 (KP836002), Dd/191/2013 (KP836006), Sc/53/2012 (KP835994), Sc/55/2012 (KP835990) and Sc/15/2007 (KP835997).



Supplementary file 2: Phylogenetic tree for the H gene nucleotidic sequences. Phylogenetic tree generated with the aligned sequences for the H gene, inferred by Bayesian methods. Sequences for the outgroup taxa were retrieved from NCBI for Pilot Whale Morbillivirus (PWMV [accession number FJ842382]); Porpoise Morbillivirus (PMV [accession number FJ648457]); Phocine Distemper Virus (PDV [accession number KC802221]); Canine Distemper Virus (CDV [accession number AY649446]) and Measles Virus (MV [accession number NC001498]). Three DMV sequences from the 90's were retrieved from NCBI and included in the trees (Z36778; AJ224705 and AJ608288) along with two sequences from 2007 (HQ829972 and HQ829973). Sequences obtained for the H gene of Portuguese and Galician isolates were also included for animals Sc/31/2009 (KT878652), Sc/290/2014 (KT878651), Sc/221/2012 (KT878650), Sc/55/2012 (KP835989), Sc/11/2013 (KT878649), Dd/302/2012 (KP836001), Dd/191/2013 (KP836005), Sc/257/2011 (KP835985), Sc/53/2012 (KP835993), and Sc/15/2007 (KP835996).



Supplementary file 2: Phylogenetic tree for the N gene nucleotidic sequences. Phylogenetic tree generated with the aligned sequences for the N gene, inferred by Bayesian methods. Sequences for the outgroup taxa were retrieved from NCBI for Pilot Whale Morbillivirus (PWMV [accession number FJ842380]); Porpoise Morbillivirus (PMV [accession number X84739]); Phocine Distemper Virus (PDV [accession number KC802221]); Canine Distemper Virus (CDV [accession number AY649446]) and Measles Virus (MV [accession number NC001498]). Two sequences from 2007 (HQ829972 and HQ829973) and one from the 90's (AJ608288) were also included in this tree. Sequences obtained for the N gene of Portuguese and Galician isolates were also included for animals Sc/15/2007 (KP835998), Dd/302/2012 (KP836000), Sc/257/2011 (KP835984), Dd/191/2013 (KP836004), Sc/53/2012 (KP835992), Sc/55/2012 (KP835988), Sc/11/2013 (KT878653), Sc/290/2014 (KT878655) and Sc/221/2012 (KT878654).

Supplementary file 4: Stranded animals tested for herpesvirus: ID code, stranding date, species, carcass condition (1-5), sex and age class.

ID CODE	*DATE OF STRANDING*	*SPECIES*	*CARCASS CONDITION*	*SEX*	*AGE CLASS*	*OBSERVATIONS*
DD-100-2011	07/01/2011	*Delphinus delphis*	3	Male	JUVENILE	
DD-101-2011	09/01/2011	*Delphinus delphis*	3	Female	JUVENILE	
DD-103-2011	11/01/2011	*Delphinus delphis*	3	Female	ADULT	
DD-105-2011	16/01/2011	*Delphinus delphis*	3	Female	JUVENILE	HV+
DD-110-2011	27/01/2011	*Delphinus delphis*	3	Male	JUVENILE	
DD-111-2011	28/01/2011	*Delphinus delphis*	2	Male	JUVENILE	
DD-204-2011	28/01/2011	*Delphinus delphis*	2	Female	JUVENILE	
DD-112-2011	28/01/2011	*Delphinus delphis*	3	Female	ADULT	HV+
DD-113-2011	01/02/2011	*Delphinus delphis*	3	Male	JUVENILE	
DD-114-2011	05/02/2011	*Delphinus delphis*	3	Male	JUVENILE	
DD-115-2011	06/02/2011	*Delphinus delphis*	3	Male	JUVENILE	
DD-116-2011	07/02/2011	*Delphinus delphis*	3	Female	JUVENILE	
DD-117-2011	15/02/2011	*Delphinus delphis*	3	Female	JUVENILE	
DD-126-2011	07/03/2011	*Delphinus delphis*	2	Female	JUVENILE	
DD-125-2011	07/03/2011	*Delphinus delphis*	2	Female	JUVENILE	
DD-127-2011	16/03/2011	*Delphinus delphis*	2	Female	JUVENILE	
DD-128-2011	16/03/2011	*Delphinus delphis*	3	Female	JUVENILE	
DD-129-2011	26/03/2011	*Delphinus delphis*	2	Female	ADULT	
DD-132-2011	30/03/2011	*Delphinus delphis*	3	Male	JUVENILE	HV+
DD-133-2011	30/03/2011	*Delphinus delphis*	3	Female	JUVENILE	
DD-134-2011	01/04/2011	*Delphinus delphis*	3	Female	JUVENILE	
dd-206-2011	02/04/2011	*Delphinus delphis*	3	Male	JUVENILE	
DD-137-2011	05/04/2011	*Delphinus delphis*	3	Female	ADULT	
DD-141-2011	06/04/2011	*Delphinus delphis*	3	Male	JUVENILE	HV+
DD-144-2011	06/04/2011	*Delphinus delphis*	3	Male	JUVENILE	
DD-156-2011	10/04/2011	*Delphinus delphis*	3	Male	JUVENILE	
DD-160-2011	11/04/2011	*Delphinus delphis*	2	Female	JUVENILE	
DD-158-2011	11/04/2011	*Delphinus delphis*	3	Male	JUVENILE	
DD-157-2011	12/04/2011	*Delphinus delphis*	3	Male	JUVENILE	
DD-161-2011	15/04/2011	*Delphinus delphis*	2	Female	JUVENILE	
DD-162-2011	18/04/2011	*Delphinus delphis*	2	Female	JUVENILE	
DD-166-2011	19/04/2011	*Delphinus delphis*	3	Male	JUVENILE	
DD-165-2011	20/04/2011	*Delphinus delphis*	4	Female	JUVENILE	
DD-167-2011	21/04/2011	*Delphinus delphis*	3	Male	JUVENILE	
DD-169-2011	23/04/2011	*Delphinus delphis*	3	Male	JUVENILE	
DD-172-2011	29/04/2011	*Delphinus delphis*	4	Male	JUVENILE	
DD-175-2011	02/05/2011	*Delphinus delphis*	2	Male	JUVENILE	
DD-176-2011	03/05/2011	*Delphinus delphis*	3	Female	JUVENILE	
DD-179-2011	04/05/2011	*Delphinus delphis*	3	Female	JUVENILE	

DD-177-2011	04/05/2011	*Delphinus delphis*	3	Female	JUVENILE	
DD-178-2011	04/05/2011	*Delphinus delphis*	3	Male	JUVENILE	
DD-183-2011	06/05/2011	*Delphinus delphis*	2	Male	JUVENILE	HV+
DD-182-2011	06/05/2011	*Delphinus delphis*	4	Female	ADULT	
DD-186-2011	08/05/2011	*Delphinus delphis*	4	Male	JUVENILE	
DD-187-2011	09/05/2011	*Delphinus delphis*	4	Male	JUVENILE	
DD-189-2011	10/05/2011	*Delphinus delphis*	3	Female	ADULT	
DD-192-2011	10/05/2011	*Delphinus delphis*	3	Female	JUVENILE	
DD-193-2011	12/05/2011	*Delphinus delphis*	3	Male	JUVENILE	
DD-198-2011	19/05/2011	*Delphinus delphis*	2	Female	JUVENILE	
DD-250-2011	19/05/2011	*Delphinus delphis*	2	Male	JUVENILE	
DD-251-2011	19/05/2011	*Delphinus delphis*	3	Female	JUVENILE	
DD-199-2011	21/05/2011	*Delphinus delphis*	3	Female	JUVENILE	
dd-216-2011	24/05/2011	*Delphinus delphis*	2	Female	JUVENILE	
DD-258-2011	27/05/2011	*Delphinus delphis*	3	Male	ADULT	
dd-219-2011	09/06/2011	*Delphinus delphis*	3	Male	JUVENILE	
DD-261-2011	10/06/2011	*Delphinus delphis*	3	Female	ADULT	
DD-269-2011	12/07/2011	*Delphinus delphis*	3	Female	JUVENILE	
dd-223-2011	14/07/2011	*Delphinus delphis*	2	Male	ADULT	
DD-287-2011	03/10/2011	*Delphinus delphis*	3	Male	ADULT	
dd-224-2011	07/10/2011	*Delphinus delphis*	2	Male	JUVENILE	
DD-288-2011	13/10/2011	*Delphinus delphis*	3	Male	JUVENILE	
DD-290-2011	30/10/2011	*Delphinus delphis*	3	Male	ADULT	
DD-291-2011	09/11/2011	*Delphinus delphis*	2	Female	JUVENILE	
DD-293-2011	23/11/2011	*Delphinus delphis*	3	Male	ADULT	
DD-294-2011	23/11/2011	*Delphinus delphis*	3	Female	JUVENILE	
DD-297-2011	09/12/2011	*Delphinus delphis*	2	Female	JUVENILE	HV+
DD-304-2011	10/12/2011	*Delphinus delphis*	2	Female	CALF	
DD-299-2011	10/12/2011	*Delphinus delphis*	3	Female	ADULT	
DD-302-2011	12/12/2011	*Delphinus delphis*	2	Male	JUVENILE	HV+ DMV+
DD-307-2011	14/12/2011	*Delphinus delphis*	2	Male	JUVENILE	
DD-306-2011	14/12/2011	*Delphinus delphis*	2	Male	JUVENILE	
DD-308-2011	14/12/2011	*Delphinus delphis*	3	Male	ADULT	
dd-310-2011	15/12/2011	*Delphinus delphis*	3	Not determined	not determined	
DD-309-2011	15/12/2011	*Delphinus delphis*	4	Female	JUVENILE	
DD-317-2011	16/12/2011	*Delphinus delphis*	1	Female	JUVENILE	HV+
DD-316-2011	17/12/2011	*Delphinus delphis*	3	Male	ADULT	
DD-318-2011	21/12/2011	*Delphinus delphis*	2	Female	ADULT	
DD-320-2011	27/12/2011	*Delphinus delphis*	3	Male	JUVENILE	
DD-319-2011	27/12/2011	*Delphinus delphis*	3	Female	JUVENILE	
DD-321-2011	28/12/2011	*Delphinus delphis*	3	Female	ADULT	
DD-159-2012	07/02/2012	*Delphinus delphis*	3	Female	ADULT	
DD-160-2012	09/02/2012	*Delphinus delphis*	3	Female	ADULT	
DD-163-2012	16/02/2012	*Delphinus delphis*	3	Female	ADULT	
DD-164-2012	20/02/2012	*Delphinus delphis*	4	Male	JUVENILE	

DD-165-2012	21/02/2012	*Delphinus delphis*	3	Female	ADULT	
DD-167-2012	23/02/2012	*Delphinus delphis*	2	Female	JUVENILE	
DD-168-2012	23/02/2012	*Delphinus delphis*	2	Female	ADULT	
DD-169-2012	25/02/2012	*Delphinus delphis*	3	Female	JUVENILE	
DD-176-2012	02/03/2012	*Delphinus delphis*	3	Female	ADULT	
DD-178-2012	03/03/2012	*Delphinus delphis*	3	Female	JUVENILE	
DD-179-2012	03/03/2012	*Delphinus delphis*	3	Female	ADULT	
DD-186-2012	11/03/2012	*Delphinus delphis*	3	Female	JUVENILE	
DD-199-2012	16/03/2012	*Delphinus delphis*	3	Male	ADULT	
DD-200-2012	16/03/2012	*Delphinus delphis*	3	Male	ADULT	
DD-206-2012	22/03/2012	*Delphinus delphis*	2	Male	JUVENILE	HV+
DD-212-2012	22/03/2012	*Delphinus delphis*	2	Female	ADULT	
DD-213-2012	24/03/2012	*Delphinus delphis*	2	Male	JUVENILE	
DD-217-2012	31/03/2012	*Delphinus delphis*	2	Female	JUVENILE	
DD-219-2012	31/03/2012	*Delphinus delphis*	3	Female	JUVENILE	
DD-224-2012	04/04/2012	*Delphinus delphis*	3	Female	JUVENILE	
DD-230-2012	07/04/2012	*Delphinus delphis*	2	Male	JUVENILE	HV+
DD-231-2012	09/04/2012	*Delphinus delphis*	3	Female	ADULT	
DD-241-2012	23/04/2012	*Delphinus delphis*	3	Male	JUVENILE	
DD-253-2012	01/05/2012	*Delphinus delphis*	2	Male	JUVENILE	
DD-277-2012	10/09/2012	*Delphinus delphis*	2	Male	JUVENILE	
DD-278-2012	14/09/2012	*Delphinus delphis*	2	Female	JUVENILE	
DD-280-2012	27/09/2012	*Delphinus delphis*	3	Female	JUVENILE	
DD-135-2012	01/11/2012	*Delphinus delphis*	3	Female	ADULT	
DD-134-2012	01/11/2012	*Delphinus delphis*	3	Male	ADULT	
DD-296-2012	12/11/2012	*Delphinus delphis*	2	Male	JUVENILE	
DD-297-2012	12/11/2012	*Delphinus delphis*	2	Female	JUVENILE	
DD-302-2012	04/12/2012	*Delphinus delphis*	1	Female	JUVENILE	
dd-150-2013	02/01/2013	*Delphinus delphis*	2	Female	JUVENILE	
DD-151-2013	02/01/2013	*Delphinus delphis*	2	Female	ADULT	
DD-155-2013	13/01/2013	*Delphinus delphis*	2	Female	JUVENILE	
DD-156-2013	14/01/2013	*Delphinus delphis*	3	Male	JUVENILE	
DD-172-2013	11/03/2013	*Delphinus delphis*	2	Female	ADULT	
DD-188-2013	22/05/2013	*Delphinus delphis*	3	Male	JUVENILE	
DD-191-2013	02/06/2013	*Delphinus delphis*	1	Female	JUVENILE	DMV+
DD-203-2013	19/07/2013	*Delphinus delphis*	2	Male	CALF	
DD-205-2013	19/07/2013	*Delphinus delphis*	2	Female	JUVENILE	
DD-212-2013	19/07/2013	*Delphinus delphis*	2	Female	ADULT	
DD-213-2013	19/07/2013	*Delphinus delphis*	2	Female	ADULT	
DD-209-2013	19/07/2013	*Delphinus delphis*	2	Male	ADULT	
DD-211-2013	19/07/2013	*Delphinus delphis*	2	Male	ADULT	
dd-213-2014	25/03/2014	*Delphinus delphis*	3	Female	JUVENILE	
GM-272-2012	29/07/2012	*Globicephala melas*	1	Male	JUVENILE	
KB-271-2012	21/07/2012	*Kogia breviceps*	1	Male	JUVENILE	
kb-266-2014	17/05/2014	*Kogia breviceps*	1	Male	ADULT	
PP-102-2011	11/01/2011	*Phocoena phocoena*	3	Male	ADULT	

PP-135-2011	01/04/2011	*Phocoena phocoena*	3	Male	ADULT	
PP-184-2011	08/05/2011	*Phocoena phocoena*	4	Female	ADULT	
PP-191-2011	10/05/2011	*Phocoena phocoena*	3	Female	CALF	
PP-252-2011	19/05/2011	*Phocoena phocoena*	4	Female	JUVENILE	
PP-253-2011	20/05/2011	*Phocoena phocoena*	2	Female	JUVENILE	
PP-255-2011	21/05/2011	*Phocoena phocoena*	2	Female	YEARLING	
PP-223-2011	18/06/2011	*Phocoena phocoena*	4	Not determined	JUVENILE	
PP-270-2011	11/07/2011	*Phocoena phocoena*	2	Female	YEARLING	
PP-273-2011	31/07/2011	*Phocoena phocoena*	2	Female	CALF	HV+
PP-282-2011	24/08/2011	*Phocoena phocoena*	1	Female	ADULT	
PP-284-2011	27/08/2011	*Phocoena phocoena*	3	Female	ADULT	
PP-285-2011	28/08/2011	*Phocoena phocoena*	3	Female	JUVENILE	
PP-286-2011	02/10/2011	*Phocoena phocoena*	3	Female	ADULT	
PP-313-2011	14/12/2011	*Phocoena phocoena*	3	Male	JUVENILE	
PP-311-2011	15/12/2011	*Phocoena phocoena*	3	Female	JUVENILE	
PP-322-2011	29/12/2011	*Phocoena phocoena*	3	Male	YEARLING	
PP-175-2012	28/02/2012	*Phocoena phocoena*	3	Male	JUVENILE	
PP-211-2012	18/04/2012	*Phocoena phocoena*	3	Male	JUVENILE	
PP-266-2012	08/06/2012	*Phocoena phocoena*	3	Male	YEARLING	
PP-267-2012	21/06/2012	*Phocoena phocoena*	2	Female	JUVENILE	
PP-268-2012	30/06/2012	*Phocoena phocoena*	3	Male	ADULT	
PP-275-2012	20/08/2012	*Phocoena phocoena*	4	Female	ADULT	
PP-276-2012	24/08/2012	*Phocoena phocoena*	2	Female	ADULT	
PP-184-2013	12/05/2013	*Phocoena phocoena*	3	Female	JUVENILE	
PP-196-2013	19/06/2013	*Phocoena phocoena*	3	Male	JUVENILE	
pp-236-2013	21/08/2013	*Phocoena phocoena*	2	Male	ADULT	
pp-251-2013	16/10/2013	*Phocoena phocoena*	2	Female	JUVENILE	
pp-271-2013	30/12/2013	*Phocoena phocoena*	2	Female	JUVENILE	HV+

pp-205-2014	22/03/2014	*Phocoena phocoena*	3	Female	JUVENILE	
pp-227-2014	08/04/2014	*Phocoena phocoena*	3	Male	JUVENILE	
SC-119-2011	17/02/2011	*Stenella coeruleoalba*	3	Female	JUVENILE	
SC-159-2011	11/04/2011	*Stenella coeruleoalba*	3	Male	JUVENILE	
SC-257-2011	22/05/2011	*Stenella coeruleoalba*	1	Male	JUVENILE	DMV+
SC-260-2011	07/06/2011	*Stenella coeruleoalba*	2	Male	JUVENILE	
SC-298-2011	10/12/2011	*Stenella coeruleoalba*	1	Female	JUVENILE	
SC-209-2012	19/03/2012	*Stenella coeruleoalba*	3	Female	JUVENILE	
SC-210-2012	22/03/2012	*Stenella coeruleoalba*	2	Female	ADULT	
SC-251-2012	24/04/2012	*Stenella coeruleoalba*	2	Unassigned	CALF	
SC-249-2012	24/04/2012	*Stenella coeruleoalba*	3	Female	ADULT	
SC-221-2012	03/08/2012	*Stenella coeruleoalba*	1	Male	ADULT	HV+ DMV+
SC-274-2012	12/08/2012	*Stenella coeruleoalba*	3	Female	CALF	
SC-182-2013	30/04/2013	*Stenella coeruleoalba*	1	Female	YEARLING	
SC-189-2013	31/05/2013	*Stenella coeruleoalba*	1	Female	JUVENILE	HV+
SC-200-2013	02/07/2013	*Stenella coeruleoalba*	2	Male	JUVENILE	
sc-188-2014	26/02/2014	*Stenella coeruleoalba*	2	Male	JUVENILE	
sc-193-2014	03/03/2014	*Stenella coeruleoalba*	3	Male	JUVENILE	DMV+
SC-12-2014	08-03-2014	*Stenella coeruleoalba*	1	Female	JUVENILE	
TT-222-2011	19/06/2011	*Tursiops truncatus*	2	Female	ADULT	
TT-233-2012	12/04/2012	*Tursiops truncatus*	2	Male	ADULT	

Supplementary file 5: Accession numbers, identification and country of origin of the sequences used in the phylogenetic trees.

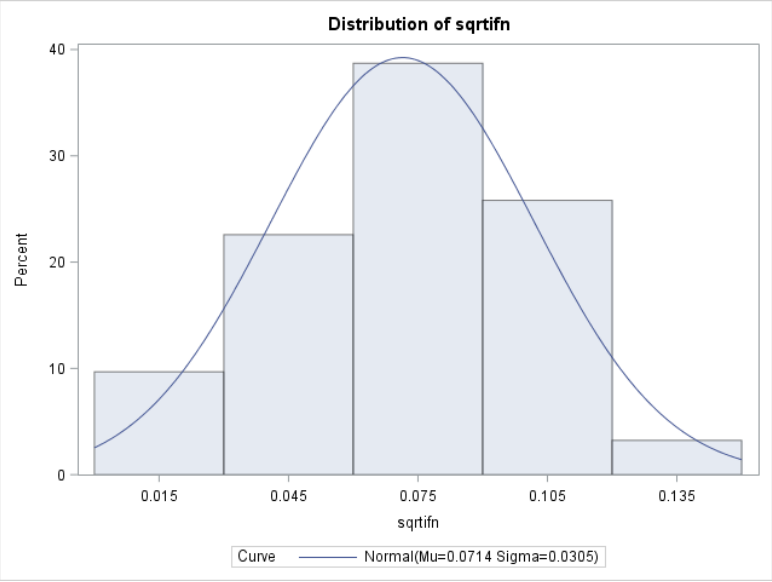
Subfamily	Accession number	Organism	Host	Country
Alphaherpesvirus	GQ888674	Unidentified herpesvirus	Stenella coeruleoalba	Spain
	AY608707	Tursiops truncatus alphaherpesvirus 1	tursiops truncatus	Germany

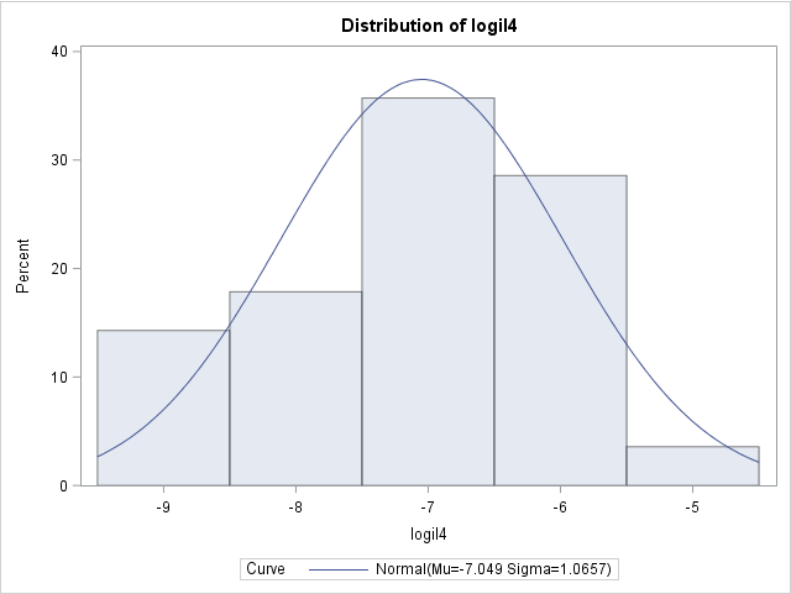
GQ429151	Delphinid herpesvirus 9	Orcinus orca	USA
GU066291	Herpesvirus whale	Ziphius cavirostris	Spain (canary islands)
GU068981	Unidentified herpesvirus	Stenella coeruleoalba	Spain
HQ214675	Unidentified herpesvirus	Stenella coeruleoalba	Spain
AY949832	Bottlenose dolphin herpesvirus	tursiops truncatus	USA
AF196646	Bottlenose dolphin herpesvirus	tursiops truncatus	USA
GQ888671	Unidentified herpesvirus	stenella coeruleoalba	Spain
JN863234	Mesoplodon densirostris herpesvirus	Mesoplodon densirostris	Spain (canary islands)
KP995686	Balaenoptera physalus alphaherpesvirus	Balaenoptera physalus	Spain
GQ888669	Unidentified herpesvirus	stenella coeruleoalba	Spain
AB510474	Melon-headed whale alphaherpesvirus	Peponocephala electra	Japan
GQ888673	Unidentified herpesvirus	stenella coeruleoalba	Spain
AF245443	Bottlenose dolphin herpesvirus	tursiops truncatus	USA
DQ295064	Tursiops truncatus alphaherpesvirus 3	tursiops truncatus	Germany
GQ429150	Delphinid herpesvirus 8	tursiops truncatus	USA
AB510473	False killer whale alphaherpesvirus	Pseudorca crassidens	Japan
GQ888675	unidentified herpesvirus	stenella coeruleoalba	Spain
GQ888670	unidentified herpesvirus	stenella coeruleoalba	Spain
DQ295063	Tursiops truncatus alphaherpesvirus 2	tursiops truncatus	Germany
AY757301	Bottlenose dolphin herpesvirus	tursiops truncatus	USA
JQ692312	Equid alphaherpesvirus 1	Ursus maritimus	Germany
KP279684	Suid alphaherpesvirus 1		China

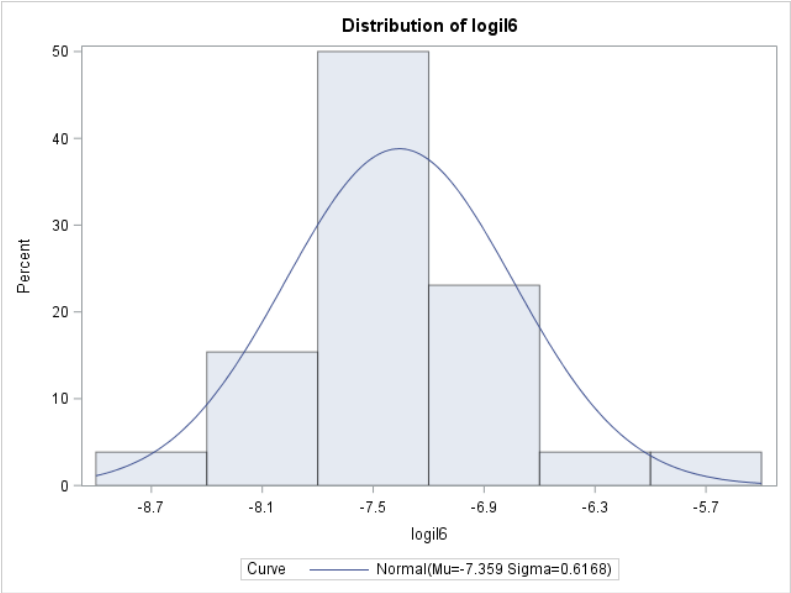
	M10792	Human alphaherpesvirus 1		
	FJ040890	Eidolon helvum simplexvirus 1	Eidolon helvum	Cameroon
	KJ995972	Columbid alphaherpesvirus 1	pigeon	China
	AF520812	Passerid herpesvirus 1	Gouldian finch	
	AY803337	Blainville's beaked whale gammaherpesvirus	Mesoplodon densirostris	USA
	AY949828	Blainville's beaked whale gammaherpesvirus	Mesoplodon densirostris	USA
	KT591613	Phocoenid herpesvirus 1	Phocoena phocoena	Netherlands
	DQ288666	Risso's dolphin gammaherpesvirus	Grampus griseus	USA
	GQ888672	unidentified herpesvirus	stenella coeruleoalba	Spain
	AY949830	Dwarf sperm whale gammaherpesvirus	Kogia simus	USA
	KP995688	Balaenoptera acutorostrata gammaherpesvirus 2	Balaenoptera acutorostrata	Spain
	AY949831	Bottlenose dolphin gammaherpesvirus	tursiops truncatus	USA
	AY952777	Bottlenose dolphin herpesvirus	tursiops truncatus	USA
Gammaherpesvirus	AY952779	Bottlenose dolphin herpesvirus	tursiops truncatus	USA
	GQ258355	Bottlenose dolphin herpesvirus	tursiops truncatus	Netherlands
	GQ258356	Bottlenose dolphin herpesvirus	tursiops truncatus	Netherlands
	GQ258353	Bottlenose dolphin herpesvirus	tursiops truncatus	Netherlands
	GQ258354	Bottlenose dolphin herpesvirus	tursiops truncatus	Netherlands
	AY952776	Atlantic bottlenose dolphin gammaherpesvirus	tursiops truncatus	USA
	DQ288667	Atlantic bottlenose dolphin gammaherpesvirus	tursiops truncatus	USA
	KP995687	Balaenoptera acutorostrata gammaherpesvirus 1	Balaenoptera acutorostrata	Spain
	KC142153	Bottlenose dolphin herpesvirus 201MG_R	tursiops truncatus	Italy
	AJ507799	Human gammaherpesvirus 4		
Betaherpesvirus	NC_001664	Human betaherpesvirus 6A	Homo sapiens	Uganda

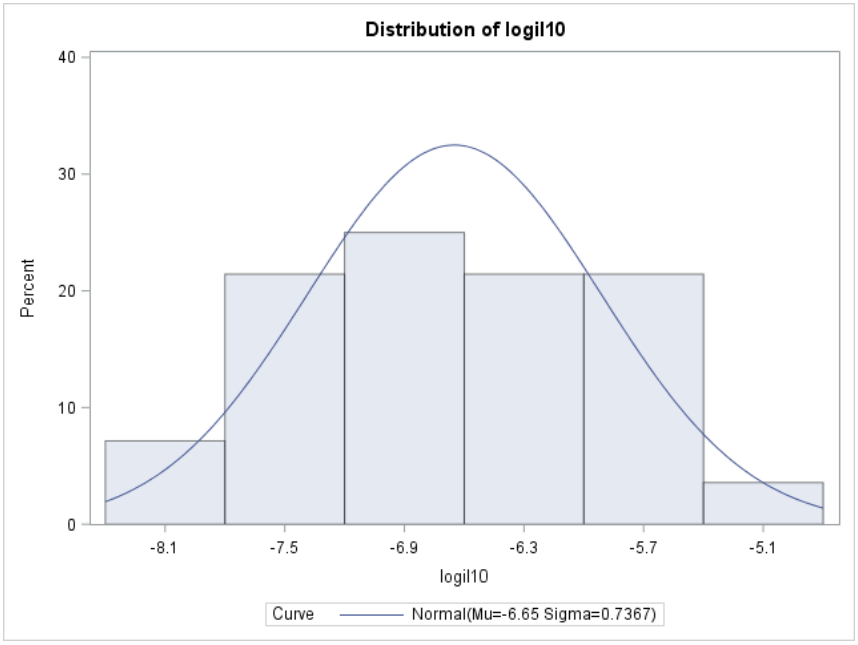
Supplementary file 5: Results obtained for the Shapiro-Wilk statistic and respective histograms. Marked in green are the accepted transformation. Histograms for the log transformation of IL4, IL6, IL10, TNF, and IL1β and for the sqrt transformation of IFN and IL12.

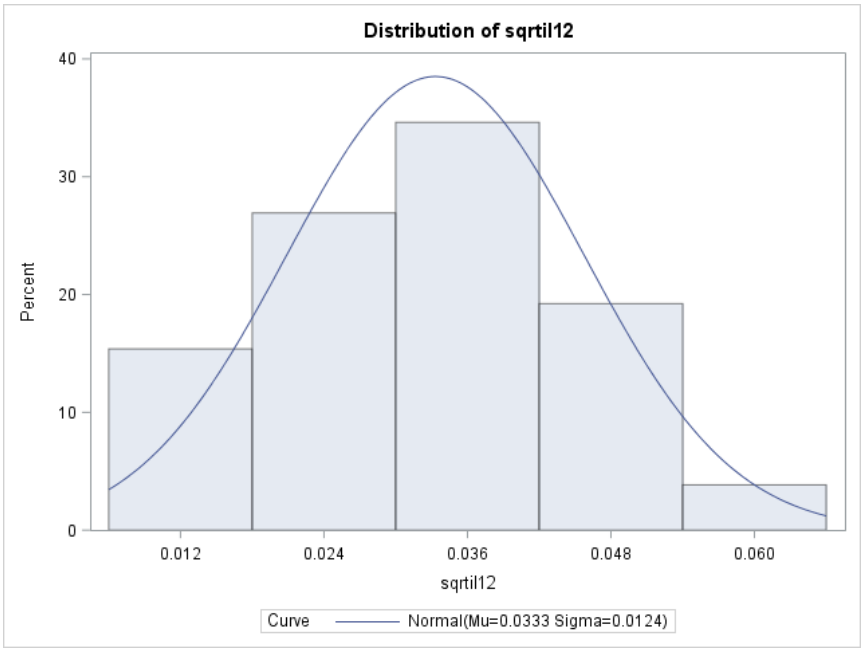
transformation	statistic W	p value (pr<W)	transformation	statistic W	p value (pr<W)
IFN	0,941	0,0893	**IL4**	0,746	<0,0001
logIFN	0,879	0,0022	**logIL4**	**0,961**	**0,3725**
loginvIFN	0,879	0,0022	**loginvIL4**	0,961	0,3725
sqrtIFN	**0,969**	**0,5004**	**sqrtIL4**	0,935	0,0843
IL6	0,642	<0,0001	**IL10**	0,766	<0,0001
logIL6	**0,940**	**0,1388**	**logIL10**	**0,979**	**0,8350**
loginvIL6	0,941	0,1388	**loginvIL10**	0,979	0,8350
sqrtIL6	0,814	0,0003	**sqrtIL10**	0,918	0,0317
IL12	0,896	0,0127	**TNF**	0,856	0,0012
logIL12	0,936	0,1059	**logTNF**	**0,950**	**0,1985**
loginvIL12	0,936	0,1059	**loginvTNF**	0,950	0,1985
sqrtIL12	**0,979**	**0,8562**	**sqrtTNF**	0,943	0,1307
IL1β	0,819	0,0003			
logIL1β	**0,978**	**0,8214**			
loginvIL1β	0,978	0,8214			
sqrtIL1β	0,943	0,1418			

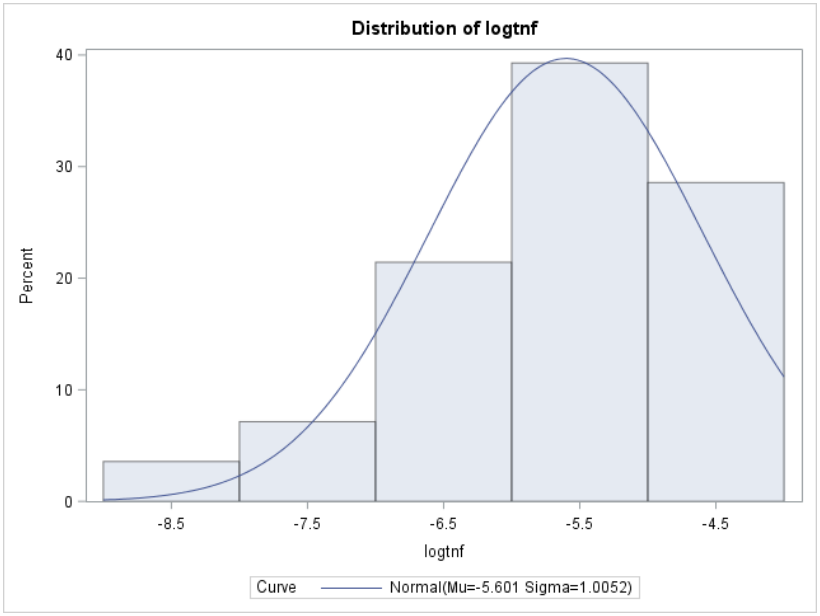


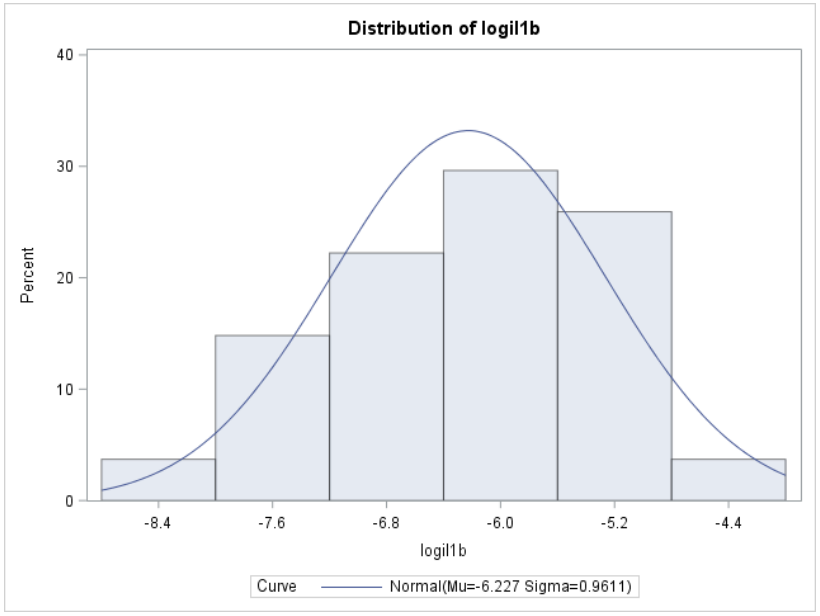

Distribution of logil4
Percent
40
30
20
10
0
-9
-8
-7
-6
-5
logil4
Curve
Normal(Mu=-7.049 Sigma=1.0657)


Distribution of logil6
Percent
50
40
30
20
10
0
-8.7
-8.1
-7.5
-6.9
-6.3
-5.7
logil6
Curve
Normal(Mu=-7.359 Sigma=0.6168)


Distribution of logil10
Percent
40
30
20
10
0
-8.1
-7.5
-6.9
-6.3
-5.7
-5.1
logil10
Curve
Normal(Mu=-6.65 Sigma=0.7367)


Distribution of sqrtil12
Percent
40
30
20
10
0
0.012
0.024
0.036
0.048
0.060
sqrtil12
Curve
Normal(Mu=0.0333 Sigma=0.0124)


Distribution of logtnf
Percent
40
30
20
10
0
-8.5
-7.5
-6.5
-5.5
-4.5
logtnf
Curve
Normal(Mu=-5.601 Sigma=1.0052)


Distribution of logil1b
Percent
40
30
20
10
0
-8.4
-7.6
-6.8
-6.0
-5.2
-4.4
logil1b
Curve
Normal(Mu=-6.227 Sigma=0.9611)

Appendix 2 – Authors' consent

Identificação do co-autor		
Hélder Eduardo Fernandes Araújo		
CC: **11793392**	**Telefone** **916518890**	**Correio eletrónico** helder.araujo@ua.pt

Tese de dissertação de Doutoramento
Título: EVALUATION OF MORBILLIVIRUS AND HERPESVIRUS INFECTION IN CETACEANS STRANDED ALONG THE PORTUGUESE COASTLINE
Autor: Maria Carolina Rocha de Medeiros Bento
Financiamento: Project CetSenti (RECI/AAG-GLO/0470/2012) (funded by Fundação Ciência e Tecnologia, I.P.) CIISA (UID/CVT/ 00276/2013) Projeto LIFE MarPro LIFE09 NAT/PT/000038 (co-funded by the European Commission

Capítulo da tese
Chapter IV: Health assessment in common dolphins: disease associated shifts in cytokine mRNA expression

Autorização
Autorizo que o capítulo anterior, seja apresentado como parte da Tese de Dissertação de Doutoramento. Lisboa, 19 de janeiro de 2023 Assinatura Assinado por: **Hélder Eduardo Fernandes Araújo** Num. de Identificação: 11793392 Data: 2023.01.19 13:25:18 +0000

Autorização dos co-autores dos trabalhos de investigação de modo a que os mesmos possam fazer parte do documento

Identificação do co-autor		
Luis Manuel Morgado Tavares		
CC: 5032406	**Telefone** 967864489	**Correio eletrónico** ltavares@fmv.ulisboa.pt

Tese de dissertação de Doutoramento
Título: EVALUATION OF MORBILLIVIRUS AND HERPESVIRUS INFECTION IN CETACEANS STRANDED ALONG THE PORTUGUESE COASTLINE
Autor: Maria Carolina Rocha de Medeiros Bento
Financiamento: Project CetSenti (RECI/AAG-GLO/0470/2012) (funded by Fundação Ciência e Tecnologia, I.P.) CIISA (UID/CVT/ 00276/2013) Projeto LIFE MarPro LIFE09 NAT/PT/000038 (co-funded by the European Commission

Capítulo da tese
Chapter IV: Health assessment in common dolphins: disease associated shifts in cytokine mRNA expression

Autorização
Autorizo que o capítulo acima indicado, seja apresentado como parte da Tese de Dissertação de Doutoramento. Lisboa, 19 de janeiro de 2023 Assinatura 

Identificação do co-autor		
Catarina Eira		
CC: 10547935	**Telefone** 915056666	**Correio eletrónico** catarina.eira@ua.pt

Tese de dissertação de Doutoramento
Título: EVALUATION OF MORBILLIVIRUS AND HERPESVIRUS INFECTION IN CETACEANS STRANDED ALONG THE PORTUGUESE COASTLINE
Autor: Maria Carolina Rocha de Medeiros Bento
Financiamento: Project CetSenti (RECI/AAG-GLO/0470/2012) (funded by Fundação Ciência e Tecnologia, I.P.) CIISA (UID/CVT/ 00276/2013) Projeto LIFE MarPro LIFE09 NAT/PT/000038 (co-funded by the European Commission

Capítulo da tese
Chapter IV: Health assessment in common dolphins: disease associated shifts in cytokine mRNA expression

Autorização
Autorizo que o capítulo anterior, seja apresentado como parte da Tese de Dissertação de Doutoramento. Lisboa, 19 de janeiro de 2023 Assinatura Assinado por: **Catarina Isabel da Costa Simões Eira** Num. de Identificação: 10547935 Data: 2023.01.19 13:45:15 +0000

Identificação do co-autor		
CC: 11167543	**Telefone** **918709008**	**Correio eletrónico** mctferreira@socpvs.org

Tese de dissertação de Doutoramento

Título:
EVALUATION OF MORBILLIVIRUS AND HERPESVIRUS INFECTION IN CETACEANS STRANDED ALONG THE PORTUGUESE COASTLINE

Autor:
Maria Carolina Rocha de Medeiros Bento

Financiamento:
Project CetSenti (RECI/AAG-GLO/0470/2012) (funded by Fundação Ciência e Tecnologia, I.P.)
CIISA (UID/CVT/ 00276/2013)
Projeto LIFE MarPro LIFE09 NAT/PT/000038 (co-funded by the European Commission

Capítulo da tese

Chapter IV: Health assessment in common dolphins: disease associated shifts in cytokine mRNA expression

Autorização

Autorizo que o capítulo anterior, seja apresentado como parte da Tese de Dissertação de Doutoramento.

Quiaios, 19 de janeiro de 2023

Assin

Assinado por: Marisa Cláudia Teixeira Ferreira
Identificação: BI11167543
Data: 2023-01-19 às 19:12:10

Identificação do co-autor		
Pedro Miguel Verdelho Gonçalves		
CC: **13975594**	**Telefone:** **912430931**	**Correio eletrónico:** **pedro_mvg_11@hotmail.com**

Tese de dissertação de Doutoramento
Título: EVALUATION OF MORBILLIVIRUS AND HERPESVIRUS INFECTION IN CETACEANS STRANDED ALONG THE PORTUGUESE COASTLINE
Autor: Maria Carolina Rocha de Medeiros Bento
Financiamento: Project CetSenti (RECI/AAG-GLO/0470/2012) (funded by Fundação Ciência e Tecnologia, I.P.) CIISA (UID/CVT/ 00276/2013) Projeto LIFE MarPro LIFE09 NAT/PT/000038 (co-funded by the European Commission

Capítulo da tese
Chapter IV: Health assessment in common dolphins: disease associated shifts in cytokine mRNA expression

Autorização
Autorizo que o capítulo anterior, seja apresentado como parte da Tese de Dissertação de Doutoramento. Lisboa, 19 de janeiro de 2023 Assinatura 

Identificação do co-autor		
Ana Isabel Simões Pereira Duarte		
CC: 5489996	**Telefone** +351-964 269 893	**Correio eletrónico** ana.duarte@iniav.pt

Tese de dissertação de Doutoramento
Título: EVALUATION OF MORBILLIVIRUS AND HERPESVIRUS INFECTION IN CETACEANS STRANDED ALONG THE PORTUGUESE COASTLINE
Autor: Maria Carolina Rocha de Medeiros Bento
Financiamento: Project CetSenti (RECI/AAG-GLO/0470/2012) (funded by Fundação Ciência e Tecnologia, I.P.) CIISA (UID/CVT/ 00276/2013) Projeto LIFE MarPro LIFE09 NAT/PT/000038 (co-funded by the European Commission

Capítulo da tese
Chapter IV: Health assessment in common dolphins: disease associated shifts in cytokine mRNA expression

Autorização
Autorizo que o capítulo anterior, seja apresentado como parte da Tese de Dissertação de Doutoramento. Lisboa, 19 de janeiro de 2023 Assinatura (Ana Isabel S.P. Duarte)

References

Adombi CM, Lelenta M, Lamien CE, Shamaki D, Koffi YM, Traoré A, Silber R, Couacy-Hymann E, Bodjo SC, Djaman JA, et al. 2011. Monkey CV1 cell line expressing the sheep–goat SLAM protein: A highly sensitive cell line for the isolation of peste des petits ruminants virus from pathological specimens. J Virol Methods. 173(2):306–313. doi:10.1016/j.jviromet.2011.02.024.

Aguilar, Alex, and J. Antonio Raga. “The Striped Dolphin Epizootic in the Mediterranean Sea.” *Ambio* 22, no. 8 (1993): 524–28. http://www.jstor.org/stable/4314142.

.

Aguirre AA, Tabor G. 2004. Introduction: Marine Vertebrates as Sentinels of Marine Ecosystem Health. Ecohealth. 1(3). doi:10.1007/s10393-004-0091-9.

Akdis CA, Blaser K. 2001. Mechanisms of interleukin-10-mediated immune suppression. Immunology. 103(2):131–136. doi:10.1046/j.1365-2567.2001.01235.x.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol. 215(3):403–410. doi:10.1016/S0022-2836(05)80360-2.

Appel MJ. 1969. Pathogenesis of canine distemper. Am J Vet Res. 30(7):1167–82.

Arbelo M, Bellière EN, Sierra E, Sacchinni S, Esperón F, Andrada M, Rivero M, Diaz-Delgado J, Fernández A. 2012. Herpes virus infection associated with interstitial nephritis in a beaked whale (Mesoplodon densirostris). BMC Vet Res. 8:243. doi:10.1186/1746-6148-8-243.

Arbelo M, Sierra E, Esperón F, Watanabe TTN, Bellière EN, Espinosa de los Monteros A, Fernández A. 2010. Herpesvirus infection with severe lymphoid necrosis affecting a beaked whale stranded in the Canary Islands. Dis Aquat Organ. 89(3):261–4. doi:10.3354/dao02208.

Augusto, J.F., Rachinas-Lopes, P. & dos Santos, M.E. (2012) Social structure of the declining resident community of common bottlenose dolphins in the Sado Estuary, Portugal. Journal of the Marine Biological Association of the United Kingdom, 92(8), 1773–1782.

Avota E, Gassert E, Schneider-Schaulies S. 2010. Measles virus-induced immunosuppression: from eVectors to mechanisms. Med Microbiol Immunol. 199:227–237. doi:10.1007/s00430-010-0152-3.

Azab W, Dayaram A, Greenwood AD, Osterrieder N. 2018. Annual Review of Virology How Host Specific Are Herpesviruses? Lessons from Herpesviruses Infecting Wild and Endangered Mammals. doi:10.1146/annurev-virology.

Banlunara W, Techangamsuwan S, Pirarat N, Kaewamatawong T, Piewbang C, Kesdangsakonwut S, Haetrakul T, Singkhum N, Chansue N, Miller M, et al. 2019. Epizootic of multi-centric, squamous cell carcinomas in populations of Indo-Pacific humpbacked dolphins Sousa chinensis in Thai waters. Dis Aquat Organ. 134(2):99–106. doi:10.3354/dao03346.

Banyard AC, Grant RJ, Romero CH, Barrett T. 2008. Sequence of the nucleocapsid gene and genome and antigenome promoters for an isolate of porpoise morbillivirus. Virus Res. 132(1–2):213–219. doi:10.1016/j.virusres.2007.11.003.

Baron MD. 2005. Wild-type Rinderpest virus uses SLAM (CD150) as its receptor. J Gen Virol. 86(6):1753–1757. doi:10.1099/vir.0.80836-0.

Barr B, Dunn JL, Daniel MD, Banford a. 1989. Herpes-like viral dermatitis in a beluga whale (Delphinapterus leucas). J Wildl Dis. 25(4):608–611.

Barrett T, Visser IK, Mamaev L, Goatley L, Van Bressem MF, Osterhaus ADME. 1993. Dolphin and Porpoise Morbillivirus are Genetically Distinct from Phocine Distemper Virus. Virology. 193:1010–1012.

Bearzi G. 2000. First report of a common dolphin (Delphinus delphis) death following penetration of a biopsy dart. undefined.

Beineke A, Puff C, Seehusen F, Baumgärtner W. 2009. Pathogenesis and immunopathology of systemic and nervous canine distemper. Vet Immunol Immunopathol. 127(1–2):1–18. doi:10.1016/j.vetimm.2008.09.023.

Beineke A, Siebert U, Müller G, Baumgärtner W. 2007. Increased blood interleukin-10 mRNA levels in diseased free-ranging harbor porpoises (Phocoena phocoena). Vet Immunol

Immunopathol. 115(1–2):100–6. doi:10.1016/j.vetimm.2006.09.006.

Beineke A, Siebert U, Wohlsein P, Baumgärtner W. 2010. Immunology of whales and dolphins. Vet Immunol Immunopathol. 133(2–4):81–94. doi:10.1016/j.vetimm.2009.06.019.

Bellehumeur C, Romero CH, Provost C, Nielsen O, Carl a, Lair S, Romero CH, Provost C, Nielsen O, Gagnon C a. 2015. Identification of a Novel Herpesvirus Associated with a Penile Proliferative Lesion in a Beluga (Delphinapterus leucas). J Wildl Dis. 51(1). doi:10.7589/2013-11-293.

Bellière EN, Esperón F, Arbelo M, Muñoz MJ, Fernández A, Sánchez-Vizcaíno JM. 2010. Presence of herpesvirus in striped dolphins stranded during the cetacean morbillivirus epizootic along the Mediterranean Spanish coast in 2007. Arch Virol. 155(8):1307–11. doi:10.1007/s00705-010-0697-x.

Bellière E N, Esperón F, Fernández a, Arbelo M, Muñoz MJ, Sánchez-Vizcaíno JM. 2011. Phylogenetic analysis of a new Cetacean morbillivirus from a short-finned pilot whale stranded in the Canary Islands. Res Vet Sci. 90(2):324–8. doi:10.1016/j.rvsc.2010.05.038.

Bellière Edwige Nina, Esperón F, Sánchez-Vizcaíno JM. 2011. Genetic comparison among dolphin morbillivirus in the 1990-1992 and 2006-2008 Mediterranean outbreaks. Infect Genet Evol. 11(8):1913–20. doi:10.1016/j.meegid.2011.08.018.

Bellini WJ, Englund G, Rozenblatt S, Arnheiter H, Richardson CD. 1985. Measles virus P gene codes for two proteins. J Virol. 53(3):908–19.

Bengtson JL, Boveng P, Franzen U, Have P, Heide-Jorgensen MP, Harkonen TJ. 1991. ANTIBODIES TO CANINE DISTEMPER VIRUS IN ANTARCTIC SEALS. Mar Mammal Sci. 7(1):85–87. doi:10.1111/j.1748-7692.1991.tb00553.x.

Bento C. 2011. Avaliação da infecção por morbilivírus em cetáceos arrojados na costa portuguesa. [dissertação de mestrado] Faculdade de Medicina Veterinária - Universidade Técnica de Lisboa.

Bento MC, Canha R, Eira C, Vingada J, Nicolau L, Ferreira M, Domingo M, Tavares L, Duarte A. 2019a. Herpesvirus infection in marine mammals: A retrospective molecular survey of stranded cetaceans in the Portuguese coastline. Infect Genet Evol. 67:222–233.

doi:10.1016/J.MEEGID.2018.11.013.

Bento MC, Canha R, Eira C, Vingada J, Nicolau L, Ferreira M, Domingo M, Tavares L, Duarte A. 2019b. Herpesvirus infection in marine mammals: A retrospective molecular survey of stranded cetaceans in the Portuguese coastline. Infect Genet Evol. 67. doi:10.1016/j.meegid.2018.11.013.

Berger A. 2000. Science commentary: Th1 and Th2 responses: What are they? Br Med J. 321(7258):424. doi:10.1136/bmj.321.7258.424.

Bernaldo de Quirós Y, González-Díaz O, Saavedra P, Arbelo M, Sierra E, Sacchini S, Jepson PD, Mazzariol S, Di Guardo G, Fernández A. 2011. Methodology for in situ gas sampling, transport and laboratory analysis of gases from stranded cetaceans. Sci Rep. 1:193. doi:10.1038/srep00193.

van Beurden SJ, IJsseldijk LL, Ordonez SR, Förster C, de Vrieze G, Gröne A, Verheije MH, Kik M. 2015. Identification of a novel gammaherpesvirus associated with (muco)cutaneous lesions in harbour porpoises (Phocoena phocoena). Arch Virol. 160(12):3115–3120. doi:10.1007/s00705-015-2607-8.

Birkun A, Kuiken T, Krivokhizhin S, Haines DM, Osterhaus ADME, Van De Bildt MWG, Joiris CR, Siebert U. 1999. Epizootic of morbilliviral disease in common dolphins (Delphinus delphis ponticus) from the Black Sea. Vet Rec. 144(4):85–92. doi:10.1136/vr.144.4.85.

Birkun Jr., A.A. 2008. *Delphinus delphis* ssp. *ponticus*. *The IUCN Red List of Threatened Species* 2008: e.T133729A3875256. https://dx.doi.org/10.2305/IUCN.UK.2008.RLTS.T133729A3875256.en.

Blanchard TW, Santiago NT, Lipscomb TP, Garber RL, McFee WE, Knowles S. 2001. Two novel alphaherpesviruses associated with fatal disseminated infections in Atlantic bottlenose dolphins. J Wildl Dis. 37(2):297–305. doi:10.7589/0090-3558-37.2.297.

Bossart GD. 2007. Emerging Diseases in Marine Mammals: from Dolphins to Manatees. Microbe. 2(11):544–549.

Bossart G D. 2011a. Marine mammals as sentinel species for oceans and human health. Vet Pathol. 48(3):676–90. doi:10.1177/0300985810388525.

Bourret V, Macé M, Bonhomme M, Crouau-roy B. 2008. Microsatellites in Cetaceans : An Overview. Open Mar Biol J. 2:38–42.

Bourret VJR, Macé MRJM, Crouau-Roy B. 2007. Genetic variation and population structure of western Mediterranean and northern Atlantic Stenella coeruleoalba populations inferred from microsatellite data. J Mar Biol Assoc UK. 87(01):265. doi:10.1017/S0025315407054859.

Van Bressem M-F, Duignan P, Banyard A, Barbieri M, Colegrove K, De Guise S, Di Guardo G, Dobson A, Domingo M, Fauquier D, et al. 2014. Cetacean Morbillivirus: Current Knowledge and Future Directions. Viruses. 6(12):5145–5181. doi:10.3390/v6125145.

Van Bressem M-F, Raga JA, Di Guardo G, Jepson PD, Duignan PJ, Siebert U, Barrett T, Santos MCDO, Moreno IB, Siciliano S, et al. 2009. Emerging infectious diseases in cetaceans worldwide and the possible role of environmental stressors. Dis Aquat Organ. 86(2):143–57. doi:10.3354/dao02101.

Van Bressem M-F, Van Waerebeek K, Garcia-Godos A, Dekegel D, Pastoret PP. 1994. Herpes-like virus in dusky dolphins, Laganorhynchus obscurus, from coastal Peru. Mar Mammal Sci. 10(3):359–368.

Bressem M Van, Raga JA, Guardo G, Jepson P, Duignan P, Barrett T, César M, Santos DO, Moreno I, Siciliano S, et al. 2009. Emerging and recurring diseases in cetaceans worldwide and the role of environmental stressors. Dis Aquat Organ. 86(2):143–157.

Van Bressem M, Visser I, Van de Bildt M, Teppema J, Raga J, Osterhaus A. 1991. Morbillivirus infection in Mediterranean striped dolphins (Stenella coeruleoalba). Vet Rec. 129(21):471–472. doi:10.1136/vr.129.21.471.

Van Bressem M, Waerebeek K V, Jepson PD, Raga J a, Duignan PJ, Nielsen O, Di Beneditto a P, Siciliano S, Ramos R, Kant W, et al. 2001. An insight into the epidemiology of dolphin morbillivirus worldwide. Vet Microbiol. 81(4):287–304.

Van Bressem MF, Gaspar R, Aznar FJ. 2003. Epidemiology of tattoo skin disease in

bottlenose dolphins Tursiops truncatus from the Sado estuary, Portugal. Dis Aquat Organ. 56(2):171–179. doi:10.3354/dao056171.

Van Bressem MF, Van Waerebeek K, Fleming M, Barrett T. 1998. Serological evidence of morbillivirus infection in small cetaceans from the Southeast Pacific. Vet Microbiol. 59(2–3):89–98. doi:10.1016/S0378-1135(97)00169-7.

Van Bressem MF, Van Waerebeek K, Raga JA. 1999. A review of virus infections of cetaceans and the potential impact of morbilliviruses , poxviruses and papillomaviruses on host population dynamics. Dis Aquat Organ. 38:53–65.

Bedjer, L., Samuels, A., Whitehead, H., Gales, N., Mann, J., Connor, R., Heithaus, M., Watson- Capps, J., Flaherty, C., Krützen, M. (2006) Decline in relative abundance of bottlenose dolphins exposed to long-term disturbance. Conservation Biology, 20(6), 1791-1798

Brito C, Sousa A. 2011. The Environmental History of Cetaceans in Portugal: Ten Centuries of Whale and Dolphin Records. Thrush S, editor. PLoS One. 6(9). doi:10.1371/journal.pone.0023951.

Buckles EL, Lowenstine LJ, Funke C, Vittore RK, Wong HN, St Leger JA, Greig DJ, Duerr RS, Gulland FMD, Stott JL. 2006. Otarine Herpesvirus-1, not Papillomavirus, is Associated with Endemic Tumours in California Sea Lions (Zalophus californianus). J Comp Pathol. 135(4):183–189. doi:10.1016/j.jcpa.2006.06.007.

Burek Kathy A., Gulland FMD, O'Hara TM. 2008. EFFECTS OF CLIMATE CHANGE ON ARCTIC MARINE MAMMAL HEALTH. Ecol Appl. 18(sp2):S126–S134. doi:10.1890/06-0553.1. [accessed 2018 Mar 27]. http://doi.wiley.com/10.1890/06-0553.1.

Cabral MJ, Almeida J, Almeida PR. 2006. Livro Vermelho dos Vertebrados de Portugal. Cabral M, ALmeida J, Almeida P, Dellinger T, Almeida F de N, M. O, editors. Habitat.:283–284.

Caignard G, Bouraï M, Jacob Y, Tangy F, Vidalain P-O. 2008. Inhibition of IFN-α/β signaling by two discrete peptides within measles virus V protein that specifically bind STAT1 and STAT2 The Infection-MAPping project I-MAP a. Virology. 383:112–120. doi:10.1016/j.virol.2008.10.014.

Cardoso TC, Ferreira HL, Okamura LH, Giroto TP, Oliveira BRSM, Fabri CUF, Gameiro R, Flores EF. 2016. Cellular response markers and cytokine gene expression in the central nervous system of cattle naturally infected with bovine herpesvirus 5. Vet J. 218:71–77. doi:10.1016/j.tvjl.2016.11.014.

Cattaneo R, Kaelin K, Baczko K, Billeter MA. 1989. Measles virus editing provides an additional cysteine-rich protein. Cell. 56(5):759–764. doi:10.1016/0092-8674(89)90679-X.

Cerón, JJ, Eckersall, PD, Martínez-Subiela S. 2005. Acute phase proteins in dogs and cats: current knowledge and future perspectives. Vet Clin Pathol. 34(2):85–99. doi:10.1111/j.1939-165X.2005.tb00019.x.

Chaussabel D. 2015. Assessment of immune status using blood transcriptomics and potential implications for global health. Semin Immunol. 27:58–66. doi:10.1016/j.smim.2015.03.002.

Childs K, Stock N, Ross C, Andrejeva J, Hilton L, Skinner M, Randall R, Goodbourn S. 2006. mda-5, but not RIG-I, is a common target for paramyxovirus V proteins. doi:10.1016/j.virol.2006.09.023.

Choi EJ, Ortega V, Aguilar HC. 2020. Feline morbillivirus, a new paramyxovirus possibly associated with feline kidney disease. Viruses. 12(5). doi:10.3390/v12050501.

Chousterman BG, Swirski FK, Weber GF. 2017. Cytokine storm and sepsis disease pathogenesis. Semin Immunopathol. 39(5):517–528. doi:10.1007/s00281-017-0639-8.

Couper KN, Blount DG, Riley EM. 2008. IL-10: The Master Regulator of Immunity to Infection. J Immunol. 180(9):5771–5777. doi:10.4049/JIMMUNOL.180.9.5771.

Connor, R.C., Wells, R.S., Mann, J., Read, A.J. (2000). The bottlenose dolphin: Social relationships in a fission-fusion society. In Mann, J., Connor, R.C., Tyack, P.L., Whitehead, H. (Eds.), Cetaceans Societies: Field Studies of Dolphins and Whales. Chicago: University of Chicago Press (pp. 91- 126).

Cruz D, Rodríquez M, Kouri V, Soto Y, Zamora L, Rodríguez D, Barrera M, Rehtanz M. 2014. Concurrent papillomavirus- and herpesvirus-infection in Atlantic bottlenose dolphins (*Tursiops truncatus*) inhabiting the Cuban coast. Mar Mammal Sci. 30(October):1564–1572. doi:10.1111/mms.12124.

Culik BM. 2011. The Toothed Whales. CMS Techni. Koen Van Waerebeek, Lima, Peru; William Perrin, La Jolla C, editor. Bonn: UNEP/CMS/ASCOBANS Secretariat. [accessed 2020 Feb 17]. www.cms.int/www.ascobans.org.

Davison AJ. 2002. Evolution of the herpesviruses. Vet Microbiol. 86:69–88.

Davison AJ. 2008. Herpesvirus: general features. In: Mahy BWJ, Van Regenmortel MH V, editors. Encyclopedia of Virology. 3.ª ed. Oxford: Academic Press - Elsevier.

Davison AJ, Eberle R, Ehlers B, Hayward GS, McGeoch DJ, Minson AC, Pellett PE, Roizman B, Studdert MJ, Thiry E. 2009. The order Herpesvirales. Arch Virol. 154(1):171–7. doi:10.1007/s00705-008-0278-4.

Delpeut S, Noyce RS, Richardson CD. 2014. The tumor-associated marker, PVRL4 (nectin-4), is the epithelial receptor for morbilliviruses. Viruses. 6(6):2268–86. doi:10.3390/v6062268.

Delves PJ, Martin SJ, Burton DR, Roitt IM (Ivan M. 2017. Roitt's essential immunology. Thirteenth edition.

Delwart E. 2012. Animal virus discovery: Improving animal health, understanding zoonoses, and opportunities for vaccine development. Curr Opin Virol. 2(3):344–352. doi:10.1016/j.coviro.2012.02.012.

Denesvre C. 2013. Marek's Disease Virus Morphogenesis. Avian Dis. 57(2s1):340–350. doi:10.1637/10375-091612-Review.1.

Díaz-Delgado J, Groch KR, Ressio R, Riskallah IPJ, Sierra E, Sacchini S, Quesada-Canales Ó, Arbelo M, Fernández A, Santos-Neto E, et al. 2019. Comparative immunopathology of cetacean morbillivirus infection in free-ranging dolphins from Western Mediterranean, Northeast-Central, and Southwestern Atlantic. Front Immunol. 10(MAR):485. doi:10.3389/fimmu.2019.00485.

Domingo M, Ferrer L, Pumarola M, Marco A, Plana J, Kennedy S, McAliskey M, Rima BK. 1990. Morbillivirus in dolphins. Nature. 348(6296):21. doi:10.1038/348021a0.

Domingo M, Vilafranca M, Visa J, Prats N, Trudgett A, Visser I. 1995. Evidence for chronic morbillivirus infection in the Mediterranean striped dolphin (Stenella coeruleoalba). Vet Microbiol. 44(2–4):229–239. doi:10.1016/0378-1135(95)00016-4.

Domingo M, Visa J, Pumarola M, Marco a. J, Ferrer L, Rabanal R, Kennedy S. 1992. Pathologic and Immunocytochemical Studies of Morbillivirus Infection in Striped Dolphins (Stenella coeruleoalba). Vet Pathol. 29(1):1–10. doi:10.1177/030098589202900101.

Duignan PJ, Geraci JR, Raga JA, Calzada N. Pathology of morbillivirus infection in striped dolphins (Stenella coeruleoalba) from Valencia and Murcia, Spain. Can J Vet Res. 1992 Jul;56(3):242-8. PMID: 1423061; PMCID: PMC1263546.

Duignan P J, House C, Geraci JR, Duffy N, Rima BK, Walsh MT, Early G, St Aubin DJ, Sadove S, Koopman H. 1995. Morbillivirus infection in cetaceans of the western Atlantic. Vet Microbiol. 44(2–4):241–9.

Duignan Padraig J., House C, Geraci JR, Early G, Copland HG, Walsh MT, Bossart GD, Cray C, Sadove S, Aubin DJ ST., et al. 1995. MORBILLIVIRUS INFECTION IN TWO SPECIES OF PILOT WHALE (GLOBICEPHALA SP.) FROM THE WESTERN ATLANTIC. Mar Mammal Sci. 11(2):150–162. doi:10.1111/j.1748-7692.1995.tb00514.x.

Duignan PJ, House C, Odell DK, Wells RS, Hansen LJ, Walsh MT, Aubin DJ St., Rima BK, Geraci JR. 2006. MORBILLIVIRUS INFECTION IN BOTTLENOSE DOLPHINS: EVIDENCE FOR RECURRENT EPIZOOTICS IN THE WESTERN ATLANTIC AND GULF OF MEXICO. Mar Mammal Sci. 12(4):499–515. doi:10.1111/j.1748-7692.1996.tb00063.x.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32(5):1792–7. doi:10.1093/nar/gkh340.

Ehlers B, Borchers K, Grund C, Frölich K, Ludwig H, Buhk HJ. 1999. Detection of new DNA polymerase genes of known and potentially novel herpesviruses by PCR with degenerate and deoxyinosine-substituted primers. Virus Genes. 18(3):211–20.

Ehlers B, Dural G, Yasmum N, Lembo T, de Thoisy B, Ryser-Degiorgis M-P, Ulrich RG, McGeoch DJ. 2008. Novel Mammalian Herpesviruses and Lineages within the Gammaherpesvirinae: Cospeciation and Interspecies Transfer. J Virol. 82(7):3509–3516. doi:10.1128/JVI.02646-07.

Ehlers B, Küchler J, Yasmum N, Dural G, Voigt S, Schmidt-Chanasit J, Jäkel T, Matuschka F-R, Richter D, Essbauer S, et al. 2007. Identification of novel rodent herpesviruses, including the first gammaherpesvirus of Mus musculus. J Virol. 81(15). doi:10.1128/JVI.00255-07.

Elk C Van, Bildt M Van De, Run P Van, Jong A De, Getu S, Verjans G, Osterhaus A, Kuiken T. 2016. Central nervous system disease and genital disease in harbor porpoises (Phocoena phocoena) are associated with different herpesviruses. Vet Res.:1–11. doi:10.1186/s13567-016-0310-8.

Elk CE Van, Jong AAW De, Osterhaus ADME, Kuiken T. 2009. GENITAL HERPESVIRUS IN BOTTLENOSE DOLPHINS (TURSIOPS TRUNCATUS): CULTIVATION, EPIDEMIOLOGY, AND ASSOCIATED PATHOLOGY. J Wildl Dis. 45(4):895–906.

Faix JD. 2013. Biomarkers of sepsis. Crit Rev Clin Lab Sci. 50(1):23–36. doi:10.3109/10408363.2013.764490.

Fenner's Veterinary Virology. Edited by N. James MacLachlan and Edward J. Dubovi. London ; Boston :Academic, 2011.

Fernández A, Esperón F, Herraéz P, de Los Monteros AE, Clavel C, Bernabé A, Sánchez-Vizcaino JM, Verborgh P, DeStephanis R, Toledano F, et al. 2008. Morbillivirus and pilot whale deaths, Mediterranean Sea. Emerg Infect Dis. 14(5):792–4. doi:10.3201/eid1405.070948.

Ferreira M, Marçalo A, Nicolau L, Pereira A, Costa E, Araújo H, Santos J, Vaqueiro J, Bento M, Gomes T, et al. 2016. Redes de arrojamentos e reabilitação: 2013-2016 . Anexo do Relatório final do projeto LIFE MarPro NAT/PT/00038.

Fickenscher H, Fleckenstein B. 2001. Herpesvirus saimiri. Philos Trans R Soc B Biol Sci. 356(1408):545–567. doi:10.1098/rstb.2000.0780.

Fiorentino David F, Bond MW, Mosmann` TR, Schumacher JH, Fong TAT, Bass H, Fiorentino D F, Leverah JA, Mosmann TR. 1989. TWO TYPES OF MOUSE T HELPER CELL IV. Th2 Clones Secrete a Factor that Inhibits Cytokine Production by Thl Clones.

doi:10.1084/jem.170.6.2081.

Fonfara S, Kakuschke a., Rosenberger T, Siebert U, Prange a. 2008. Cytokine and acute phase protein expression in blood samples of harbour seal pups. Mar Biol. 155(3):337–345. doi:10.1007/s00227-008-1031-y.

Fontaine MC, Baird SJE, Piry S, Ray N, Tolley KA, Duke S, Birkun AA, Ferreira M, Jauniaux T, Llavona Á, et al. 2007. Rise of oceanographic barriers in continuous populations of a cetacean: The genetic structure of harbour porpoises in Old World waters. BMC Biol. 5(1):1–16. doi:10.1186/1741-7007-5-30.

Fontaine MC, Roland K, Calves I, Austerlitz F, Palstra FP, Tolley KA, Ryan S, Ferreira M, Jauniaux T, Llavona A, et al. 2014. Postglacial climate changes and rise of three ecotypes of harbour porpoises, *Phocoena phocoena* , in western Palearctic waters. Mol Ecol. 23(13):3306–3321. doi:10.1111/mec.12817.

Fontaine MC, Tolley KA, Michaux JR, Birkun A, Ferreira M, Jauniaux T, Llavona Á, Öztürk B, Öztürk AA, Ridoux V, et al. 2010. Genetic and historic evidence for climate-driven population fragmentation in a top cetacean predator: the harbour porpoises in European water. Proc R Soc B Biol Sci. 277(1695):2829–2837. doi:10.1098/rspb.2010.0412.

Forcada J, Aguilar A, Hammond PS, Pastor X, Aguilar R. 1994. DISTRIBUTION and NUMBERS OF STRIPED DOLPHINS IN THE WESTERN MEDITERRANEAN SEA AFTER THE 1990 EPIZOOTIC OUTBREAK. Mar Mammal Sci. 10(2):137–150. doi:10.1111/j.1748-7692.1994.tb00256.x.

Funke C, King DP, McBain JF, Adelung D, Stott JL. 2003. Expression and functional characterization of killer whale (Orcinus orca) interleukin-6 (IL-6) and development of a competitive immunoassay. Vet Immunol Immunopathol. 93(1–2):69–79.

García-Martínez J, Moya A, Raga JA, Latorre A. 1999. Genetic differentiation in the striped dolphin Stenella coeruleoalba from European waters according to mitochondrial DNA (mtDNA) restriction analysis. Mol Ecol. 8(6):1069–1073. doi:10.1046/j.1365-294X.1999.00672.x.

Garg RK, Mahadevan A, Malhotra HS, Rizvi I, Kumar N, Uniyal R. 2019. Subacute sclerosing panencephalitis. Rev Med Virol. 29(5):e2058. doi:10.1002/rmv.2058.

Geraci JR, Lounsbury VJ. 1993. Marine Mammals Ashore: A Field Guide for Strandings. Galveston, Texas: Texas A&M Sea Grant College Program.

Gilbert J, O'Dor R, Vogel T. 2011. Survey data are still vital to science. Nature. 469:162. doi:10.1038/469162a.

Goldstein T, Gulland FMD, Braun RC, Antonelis GA, Kashinsky L, Rowles TK, Mazet JAK, Dalton LM, Aldridge BM, Stott JL. 2006. Molecular Identification of a Novel Gamma Herpesvirus in the Endangered Hawaiian Monk Seal (Monachus Schauinslandi). Mar Mammal Sci. 22(2):465–471. doi:10.1111/j.1748-7692.2006.00025.x.

Goldstein T, Lowenstine L, Lipscomb T, Keener Mazet JA. 2006. Infection with a Novel Gammaherpesvirus in Northern Elephant Seals (Mirounga angustirostris) Ecological surveillance of wildlife pathogens View project Capacity building for Ecological Surveillance of Hemorrhagic viral fevers and Molecular Diagnosis, department of Biological sciences, Njala university. View project. Artic J Wildl Dis. doi:10.7589/0090-3558-42.4.830.

Goldstein T, Lowenstine LJ, Lipscomb TP, Mazet JAK, Novak J, Stott JL, Gulland FMD. 2006. Infection with a novel gammaherpesvirus in northern elephant seals (Mirounga angustirostris). J Wildl Dis. 42(4):830–5. doi:10.7589/0090-3558-42.4.830.

Grant RJ, Banyard AC, Barrett T, Saliki JT, Romero CH. 2009. Real-time RT-PCR assays for the rapid and differential detection of dolphin and porpoise morbilliviruses. J Virol Methods. 156(1–2):117–23. doi:10.1016/j.jviromet.2008.11.008.

Griffin DE. 2010. Measles virus-induced suppression of immune responses. Immunol Rev. 236(1):176–189. doi:10.1111/j.1600-065X.2010.00925.x.

Griffin DE. 2016. The immune response in measles: Virus control, clearance and protective immunity. Viruses. 8(10). doi:10.3390/v8100282.

Grinde B. 2013. Herpesviruses: latency and reactivation - viral strategies and host response. J Oral Microbiol. 5(5):1–9. doi:10.3402/jom.v5i0.22766.

Groch KR, Colosio AC, Marcondes MCC, Zucca D, Díaz-Delgado J, Niemeyer C, Marigo J, Brandão PE, Fernández A, Catão-Dias JL. 2014. Novel cetacean morbillivirus in

Guiana Dolphin, Brazil. Emerg Infect Dis. 20(3):511–513. doi:10.3201/eid2003.131557.

Grondman I, Pirvu A, Riza A, Ioana M, Netea MG. 2020. Biomarkers of inflammation and the etiology of sepsis. Biochem Soc Trans. 48(1):1–14. doi:10.1042/BST20190029.

Di Guardo G, Di Francesco CE, Eleni C, Cocumelli C, Scholl F, Casalone C, Peletto S, Mignone W, Tittarelli C, Di Nocera F, et al. 2013. Morbillivirus infection in cetaceans stranded along the Italian coastline: pathological, immunohistochemical and biomolecular findings. Res Vet Sci. 94(1):132–7. doi:10.1016/j.rvsc.2012.07.030.

Guerra, M., Dawson, S.M., Brough, T.E., Rayment, W.J. (2014) Effects of boats on the surface and acoustic behaviour of an endangered population of bottlenose dolphins. Endangered Species Research, 24, 221-236.

Gulland FMD, Hall AJ. 2006. The role of infectious disease in influencing status and trends. In: Reynolds JE, Perrin WF, Reeves RR, Montgomery S, Ragen T, editors. Marine mammal research: conservation beyond crisis. Baltimore: Johns Hopkins University Press. p. 47–61.

Gutierrez J, Issacson RS, Koppel BS. 2010. Subacute sclerosing panencephalitis: An update. Dev Med Child Neurol. 52(10):901–907. doi:10.1111/j.1469-8749.2010.03717.x.

Haeryfar SMM. 2020. On invariant T cells and measles: A theory of “innate immune amnesia.” PLoS Pathog. 16(12). doi:10.1371/journal.ppat.1009071.

Han H, Ma Q, Li C, Liu R, Zhao L, Wang W, Zhang P, Liu X, Gao G, Liu F, et al. 2020. Profiling serum cytokines in COVID-19 patients reveals IL-6 and IL-10 are disease severity predictors. Emerg Microbes Infect. 9(1):1123–1130. doi:10.1080/22221751.2020.1770129.

Hao M, Wang X, Du J, Liu L, Jiao Y, Wu H, Zheng J, Li W. 2015. Cytokine levels are associated with the severity of varicella infections. J Infect Dev Ctries. 9(2):190–196. doi:10.3855/jidc.5255.

Harder T. C., Harder M, Vos H, Kulonen K, Kennedy-Stoskopf S, Liess B, Appel MJG, Osterhaus a. DME. 1996. Characterization of phocid herpesvirus-1 and -2 as putative alpha-

and gammaherpesviruses of North American and European pinnipeds. J Gen Virol. 77:27–35. doi:10.1099/0022-1317-77-1-27.

Harzen S, Brunnick BJ. 1997. Skin disorders in bottlenose dolphins (Tursiops truncatus), resident in the Sado estuary, Portugal. Aquatic Mammals 23.1 59-68.

Hashiguchi T, Maenaka K, Yanagi Y. 2011. Measles Virus Hemagglutinin: Structural Insights into Cell Entry and Measles Vaccine. Front Microbiol. 2(DEC):247. doi:10.3389/fmicb.2011.00247.

Hashiguchi T, Ose T, Kubota M, Maita N, Kamishikiryo J, Maenaka K, Yanagi Y. 2011. Structure of the measles virus hemagglutinin bound to its cellular receptor SLAM. Nat Struct Mol Biol. 18(2):135–142. doi:10.1038/nsmb.1969.

Headley SA, Amude AM, Alfieri AF, Bracarense APFRL, Alfieri AA, Summers BA. 2009. Molecular detection of Canine distemper virus and the immunohistochemical characterization of the neurologic lesions in naturally occurring old dog encephalitis. J Vet Diagnostic Investig. 21(5):588–597. doi:10.1177/104063870902100502.

Huelsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics. 17(8):754–5.

Hunt KE, Moore MJ, Rolland RM, Kellar NM, Hall AJ, Kershaw J, Raverty SA, Davis CE, Yeates LC, Fauquier DA, et al. 2013. Overcoming the challenges of studying conservation physiology in large whales: a review of available methods. Conserv Physiol. 1(1):cot006–cot006. doi:10.1093/conphys/cot006.

Iwasaki M, Takeda M, Shirogane Y, Nakatsu Y, Nakamura T, Yanagi Y. 2009. The Matrix Protein of Measles Virus Regulates Viral RNA Synthesis and Assembly by Interacting with the Nucleocapsid Protein Downloaded from. J Virol. 83:10374–10383. doi:10.1128/JVI.01056-09.

Jefferson TA, Leatherwood S, Webber MA. 1993. Marine mammals of the world. FAO Species Identification Guide for Fishery Purposes, editor. Rome.

Jones DT, Taylor WR, Thornton JM. 1992. The rapid generation of mutation data

matrices from protein sequences. Comput Appl Biosci. 8(3):275–82.

Kennedy MA. 2010. A Brief Review of the Basics of Immunology: The Innate and Adaptive Response. Vet Clin North Am Small Anim Pract. 40(3):369–379. doi:10.1016/J.CVSM.2010.01.003.

Kennedy S, Lindstedt IJ, McAliskey MM, McConnell SA, McCullough SJ. 1992. Herpesviral encephalitis in a harbor porpoise (Phocoena phocoena). J Zoo Wildl Med. 23(3).

Kennedy S, Smyth J a., Cush PF, McAliskey M, McCullough SJ, Rima BK. 1991. Histopathologic and Immunocytochemical Studies of Distemper in Harbor Porpoises. Vet Pathol. 28(1):1–7. doi:10.1177/030098589102800101.

Kennedy S, Smyth JA, Cush PF, McCullough SJ, Allan GM, McQuaid S. 1988. Viral distemper now found in porpoises. Nature. 336(6194):21. doi:10.1038/336021a0.

King DP, Hure MC, Goldstein T, Aldridge BM, Gulland FMD, Saliki JT, Buckles EL, Lowenstine LJ, Stott JL. 2002. Otarine herpesvirus-1: A novel gammaherpesvirus associated with urogenital carcinoma in California sea lions (Zalophus californianus). Vet Microbiol. 86:131–137. doi:10.1016/S0378-1135(01)00497-7.

King DP, Schrenzel MD, McKnight ML, Reidarson TH, Hanni KD, Stott JL, Ferrick DA. 1996. Molecular cloning and sequencing of interleukin 6 cDNA fragments from the harbor seal (Phoca vitulina), killer whale (Orcinus orca), and Southern sea otter (Enhydra lutris nereis). Immunogenetics. 43(4):190–195. doi:10.1007/BF00587299.

Klemens J, ciurkiewicz M, iseringhausen M, Klotz D, pfankuche VM, Ulrich R, Herder V, Baumgärtner W, Beineke A. neurotoxic potential of reactive astrocytes in canine distemper demyelinating leukoencephalitis. doi:10.1038/s41598-019-48146-9.

Kuiken T, Hartmann MG. 1991. Cetacean Dissection techniques and tissue sampling. In: Proceedings of the First ECS Workshop on Cetacean Pathology.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol. 33(7):1870–1874. doi:10.1093/molbev/msw054.

Lebich M, Harder TC, Frey HR, Visser IK, Osterhaus AD, Liess B. Comparative

immunological characterization of type-specific and conserved B-cell epitopes of pinniped, felid and canid herpesviruses. Arch Virol. 1994;136(3-4):335-47. doi: 10.1007/BF01321062. PMID: 7518225.

Lecis R, Tocchetti M, Rotta A, Naitana S, Ganges L, Pittau M, Alberti A. 2014. First Gammaherpesvirus Detection in a Free-Living Mediterranean Bottlenose Dolphin. J Zoo Wildl Med. 45(4):922–925. doi:10.1638/2014-0019.1.

Lemon K, de Vries RD, Mesman AW, Mcquaid S, van Amerongen G, Yuksel S, Ludlow M, Rennick LJ, Kuiken T, Rima BK, et al. 2011. Early target cells of measles virus after aerosol infection of non-human primates. PLoS Pathog. 7(1):e1001263. doi:10.1371/journal.ppat.1001263.

Lipscomb P, Yvonne F. 1994. Morbilliviral (Tursiops Disease truncatus) in Atlantic from the Bottlenose 1987-1988 Dolphins Epizootic from. J Wildl Dis. 30(4):567–571.

Lipscomb TP, Kennedy S, Moffett D, Krafft A, Klaunberg BA, Lichy JH, Regan GT, Worthy GAJ, Taubenberger JK. 1996. Morbilliviral epizootic in bottlenose dolphins of the Gulf of Mexico. J Vet Diagn Invest.(8):283–290.

Lipscomb TP, Scott DP, Garber RL, Krafft AE, Tsai MM, Lichy JH, Taubenberger JK, Schulman FY, Gulland FM. 2000. Common metastatic carcinoma of California sea lions (Zalophus californianus): evidence of genital origin and association with novel gammaherpesvirus. Vet Pathol. 37(6):609–17.

Locksley RM, Scott P. 1991. Helper T-cell subsets in mouse leishmaniasis: induction, expansion and effector function. Immunol Today. 12(3):A58–A61. doi:10.1016/S0167-5699(05)80017-9.

Ludlow M, McQuaid S, Milner D, de Swart RL, Duprex WP. 2015. Pathological consequences of systemic measles virus infection. J Pathol. 235(2):253–65. doi:10.1002/path.4457.

MacLachlan JN, Dubovi EJ, editors. 2011a. Herpesvirales. In: Fenner's veterinary virology. 4.ª ed. Londres: Academic Press - Elsevier.

MacLachlan JN, Dubovi EJ, editors. 2011b. Laboratory Diagnosis of Viral Infections.

In: Fenner's veterinary virology. 4.ª ed. Londres: Academic Press - Elsevier.

Maness HTD, Nollens HH, Jensen ED, Goldstein T, LaMere S, Childress A, Sykes J, St Leger J, Lacave G, Latson FE, et al. 2011. Phylogenetic analysis of marine mammal herpesviruses. Vet Microbiol. 149(1–2):23–9. doi:10.1016/j.vetmic.2010.09.035.

Manire CA, Smolarek KA, Romero CH, Kinsel MJ, Clauss TM, Byrd L. 2006. Proliferative Dermatitis Associated With a Novel Alphaherpesvirus in an Atlantic Bottlenose Dolphin (Tursiops Truncatus). J Zoo Wildl Med. 37(2):174–181. doi:10.1638/05-006.1.

Marpro (2012) Código de boas práticas para a mitigação de interações das pescarias portuguesas com cetáceos e aves marinhas. Life + Marpro NAT/PT/00038 36 pp.

Martineau D, Lagacé A, Béland P, Higgins R, Armstrong D, Shugart LR. 1988. Pathology of stranded beluga whales (Delphinapterus leucas) from the St. Lawrence Estuary, Québec, Canada [Abstract]. J Comp Pathol. 98(3).

Mazzariol S, Marcer F, Mignone W, Serracca L, Goria M, Marsili L, Di Guardo G, Casalone C. 2012. Dolphin Morbillivirus and Toxoplasma gondii coinfection in a Mediterranean fin whale (Balaenoptera physalus). BMC Vet Res. 8. doi:10.1186/1746-6148-8-20.

McCullough SJ, McNeilly F, Allan GM, Kennedy S, Smyth JA, Cosby SL, McQuaid S, Rima BK. 1991. Isolation and characterisation of a porpoise morbillivirus. Arch Virol. 118(3–4):247–252. doi:10.1007/BF01314034.

McGeoch DJ, Rixon FJ, Davison AJ. 2006. Topics in herpesvirus genomics and evolution. Virus Res. 117:90–104. doi:10.1016/j.virusres.2006.01.002.

Melero M, Crespo-Picazo JL, Rubio-Guerri C, García-Párraga D, Sánchez-Vizcaíno JM. 2015. First molecular determination of herpesvirus from two mysticete species stranded in the Mediterranean Sea. BMC Vet Res. 11(1):283. doi:10.1186/s12917-015-0596-1.

Melero M, García-Párraga D, Corpa JM, Ortega J, Rubio-Guerri C, Crespo JL, Rivera-Arroyo B, Sánchez-Vizcaíno JM. 2014. First molecular detection and characterization of herpesvirus and poxvirus in a Pacific walrus (Odobenus rosmarus divergens). BMC Vet Res. 10:968. doi:10.1186/s12917-014-0308-2.

Melia MM, Earle JP, Abdullah H, Reaney K, Tangy F, Cosby SL. 2014. Use of SLAM and PVRL4 and identification of Pro-HB-EGF as cell entry receptors for wild type phocine distemper virus. PLoS One. 9(8). doi:10.1371/journal.pone.0106281.

Messling V von, Svitek N, Cattaneo R. 2006. Receptor (SLAM [CD150]) Recognition and the V Protein Sustain Swift Lymphocyte-Based Invasion of Mucosal Tissue and Lymphatic Organs by a Morbillivirus. J Virol. 80(12):6084. doi:10.1128/JVI.00357-06.

Minjolle S, Michelet C, Jusselin I, Joannes M, Cartier F, Colimon R. 1999. Amplification of the six major human herpesviruses from cerebrospinal fluid by a single PCR. J Clin Microbiol. 37(4):950–953.

Molnár V, Jánoska M, Harrach B, Glávits R, Pálmai N, Rigó D, Sós E, Liptovszky M. 2008. Detection of a novel bat gammaherpesvirus in Hungary. Acta Vet Hung. 56(4):529–38. doi:10.1556/AVet.56.2008.4.10.

Moore KW, De Waal Malefyt R, Coffman RL, O'Garra A. 2001. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol. 19:683–765. doi:10.1146/annurev.immunol.19.1.683.

Moore SE. 2008. Marine mammals as ecosystem sentinels. In: Journal of Mammalogy. Vol. 89. Oxford Academic. p. 534–540.

Moss WJ, Ota MO, Griffin DE. 2004. Measles: immune suppression and immune responses. Int J Biochem Cell Biol. 36:1380–1385. doi:10.1016/j.biocel.2004.01.019.

Moss WJ, Ryon JJ, Monze M, Griffin DE, Feinstone WH. 2002. Differential Regulation of Interleukin (IL)-4, IL-5, and IL-10 during Measles in Zambian Children.

Mühlebach MD, Mateo M, Sinn PL, Prüfer S, Uhlig KM, Leonard VHJ, Navaratnarajah CK, Frenzke M, Wong XX, Sawatsky B, et al. 2011. Adherens junction protein nectin-4 is the epithelial receptor for measles virus. Nature. 480(7378):530–533. doi:10.1038/nature10639.

Müller U, Steinhoff U, Reis LFL, Hemmi S, Pavlovic J, Zinkernagel RM, Aguet M. 1994. Functional role of type I and type II interferons in antiviral defense. Science (80-). 264(5167):1918–1921. doi:10.1126/science.8009221.

Nelms SE, Alfaro-Shigueto J, Arnould JPY, Avila IC, Nash SB, Campbell E, Carter MID, Collins T, Currey RJC, Domit C, et al. 2021. Marine mammal conservation: over the horizon. Endanger Species Res. 44:291–325. doi:10.3354/ESR01115.

Noguchi K, Shimoda H, Terada Y, Shimojima M, Kohyama K, Inoshima Y, Maeda K. 2013. Isolation of a novel herpesvirus from a Pacific white-sided dolphin. Arch Virol. 158:695–699. doi:10.1007/s00705-012-1536-z.

Noyce RS, Bondre DG, Ha MN, Lin LT, Sisson G, Tsao MS, Richardson CD. 2011. Tumor cell marker pvrl4 (nectin 4) is an epithelial cell receptor for measles virus. PLoS Pathog. 7(8). doi:10.1371/journal.ppat.1002240.

O'Garra A, Vieira PL, Vieira P, Goldfeld AE. 2004. IL-10-producing and naturally occurring CD4+Tregs: Limiting collateral damage. J Clin Invest. 114(10):1372–1378. doi:10.1172/JCI23215.

O'Halloran C, McCulloch L, Rentoul L, Alexander J, Hope JC, Gunn-Moore DA. 2018. Cytokine and Chemokine Concentrations as Biomarkers of Feline Mycobacteriosis. Sci Rep. 8(1):1–11. doi:10.1038/s41598-018-35571-5.

Ohishi K, Ando A, Suzuki R, Takishita K, Kawato M, Katsumata E, Ohtsu D, Okutsu K, Tokutake K, Miyahara H, et al. 2010. Host-virus specificity of morbilliviruses predicted by structural modeling of the marine mammal SLAM, a receptor. Comp Immunol Microbiol Infect Dis. 33(3):227–241. doi:10.1016/j.cimid.2008.10.003.

Ohishi K, Suzuki R, Maruyama T. 2012. Host-Virus Specificity of the Morbillivirus Receptor, SLAM, in Marine Mammals: Risk Assessment of Infection Based on Three-Dimensional Models. New Approaches to Study Mar Mamm.(Dmv):183–204. doi:10.5772/2731.

Okada H, Kobune F, Sato TA, Kohama T, Takeuchi Y, Abe T, Takayama N, Tsuchiya T, Tashiro M. 2000. Extensive lymphopenia due to apoptosis of uninfected lymphocytes in acute measles patients. Arch Virol. 145(5):905–920. doi:10.1007/S007050050683.

Okonechnikov K, Golosova O, Fursov M, Varlamov A, Vaskin Y, Efremov I, German Grehov OG, Kandrov D, Rasputin K, Syabro M, et al. 2012. Unipro UGENE: A unified bioinformatics toolkit. Bioinformatics. 28(8):1166–1167. doi:10.1093/bioinformatics/bts091.

Osterhaus a DME, Spijkers HEM, Groen J, Teppema JS, van Steenis G. 1985. The Isolation and Partial Characterization of a Hifhly Pathoflenie Herpesvirus from the Harbor Seal (Phoca vilulina) By. Arch Virol. 251.

Osterhaus ADME, Groen J, UytdeHaag F, Visser IK, Vedder EJ, Crowther J, Bostock CJ. 1989. Morbillivirus infections in European seals before 1988. Vet Rec.(125):326.

Patel A, Rajak KK, Balamurugan V, Sen A, Sudhakar SB, Bhanuprakash V, Singh RK, Pandey AB. 2012. Cytokines expression profile and kinetics of Peste des petits ruminants virus antigen and antibody in infected and vaccinated goats. Virol Sin. 27(4):265–271. doi:10.1007/s12250-012-3240-2.

Pei C, Lei X-Y, Yuan X-P, Wang D, Zhao Q-Z, Zhang Q-Y. 2012. Herpes-like Virus Infection in Yangtze Finless Porpoise (Neophocaena phocaenoides): Pathology, Ultrastructure and Molecular Analysis. J Wildl Dis. 48(1):235–237. doi:10.7589/0090-3558-48.1.235.

Pellett PE, Roizman B. 2013. Herpesviridae. In: Knipe DM, Howley PM, editors. Field's virology. 6.ª ed. Filadelfia: Lippincott Williams & Wilkins.

Perrin WF, Wilson CE, Archer FI. 1994. Striped dolphin, Stenella coeruleoalba (Meyen, 1833). In: Ridgway SHARH, editor. Handbook of marine mammals vol 5. London: Academic Press. p. 129–159.

Porter AG, Jänicke RU. 1999. Emerging roles of caspase-3 in apoptosis. Cell Death Differ 1999 62. 6(2):99–104. doi:10.1038/sj.cdd.4400476.

Profeta F, Di Francesco CE, Marsilio F, Mignone W, Di Nocera F, De Carlo E, Lucifora G, Pietroluongo G, Baffoni M, Cocumelli C, et al. 2015. Retrospective seroepidemiological investigations against Morbillivirus, Toxoplasma gondii and Brucella spp. in cetaceans stranded along the Italian coastline (1998-2014). Res Vet Sci. 101:89–92. doi:10.1016/J.RVSC.2015.06.008.

Raga J, Banyard A, Domingo M, Corteyn M, Bressem M Van, Fernández M, Aznar F, Barrett T. 2008. Morbillivirus. Emerg Infect Dis. 14(3):471–473.

Raga JA, Banyard A, Domingo M, Corteyn M, Van Bressem M-F, Fernández M,

Aznar FJ, Barrett T. 2008. Dolphin morbillivirus epizootic resurgence, Mediterranean Sea. Emerg Infect Dis. 14(3):471–473. doi:10.3201/eid1403.071230.

Ramachandran A, Parisien J-P, Horvath CM. 2008. STAT2 Is a Primary Target for Measles Virus V Protein-Mediated Alpha/Beta Interferon Signaling Inhibition Downloaded from. J Virol. 82(17):8330–8338. doi:10.1128/JVI.00831-08.

Ramshaw IA, Ramsay A, Karupiah G, Rolph MS, Mahalingam S, Ruby JC, Rflmshaw' L, Aliswirj R, Guii<i,' :Egamii Karupiah' MS, Suraidmn Myhoimgum' R'. 1997. Cytokines and immunity to viral infections Authors' addresses. Immunol Rev. 159:119–135.

Reddy ML, Dierauf LA, Gulland FMD. 2001. Marine Mammals as Sentinels of Ocean Health. In: Dierauf LA, Gullan FMD, editors. CRC Handbook of Marine Mammal Medicine. 2.ª ed. Boca Raton (Florida): CRC Press.

Reid JB, Reid JB, Evans PGH, Evans PGH, Northridge SP, Northridge SP. 2003. Atlas of Cetacean distribution in north-west European waters. Jt Nat Conserv Comm. 81(2):117–27. doi:10.1017/S0022149X07754718.

Reynolds III, J.E., Wells, R.S. & Eide, S. D. (2000) The bottlenose dolphin: Biology and conservation. Gainesville: University Press of Florida.

Reyes JC. 1991. The conservation of small cetaceans: a review. Rep Prep Secr Conv Conserv Migr species wild Anim UNEP/CMS Secr Bonn.

Resolução do conselho de ministros nº68/2021 de 4 de Junho, Estratégia nacional para o mar 2021-2030. Diário de República nº108/2021 - SérieI. Presidência do Conselho de Ministros.

Rice D. 1998. Marine Mammals of the World Systematics and Distribution. In: Special Publication number 4: The Society for Marine Mammalogy. p. 231 p.

Rigopoulou EI, Abbott WGH, Haigh P, Naoumov N V. 2005. Blocking of interleukin-10 receptor - A novel approach to stimulate T-helper cell type 1 responses to hepatitis C virus. Clin Immunol. 117(1):57–64. doi:10.1016/j.clim.2005.06.003.

Rocha De Medeiros Bento MC, Costa CI, Eira S, Vingada JV, Marçalo AL, Cláudia M,

Ferreira T, Lopez Fernandez A, Manuel L, Tavares M, et al. 2016. New insight into dolphin morbillivirus phylogeny and epidemiology in the northeast Atlantic: opportunistic study in cetaceans stranded along the Portuguese and Galician coasts. BMC Vet Res. 12. doi:10.1186/s12917-016-0795-4.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 19(12):1572–4.

Rosel PE, France SC, Wang JY, Kocher TD. 1999. Genetic structure of harbour porpoise Phocoena phocoena populations in the northwest Atlantic based on mitochondrial and nuclear markers. In: Molecular Ecology. Vol. 8. Blackwell Publishing Ltd. p. S41–S54.

Roth J, De Souza GEP. 2001. Fever induction pathways: Evidence from responses to systemic or local cytokine formation. Brazilian J Med Biol Res. 34(3):301–314. doi:10.1590/S0100-879X2001000300003.

Rubio-Guerri C, Melero M, Esperón F, Bellière EN, Arbelo M, Crespo JL, Sierra E, García-Párraga D, Sánchez-Vizcaíno JM. 2013. Unusual striped dolphin mass mortality episode related to cetacean morbillivirus in the Spanish Mediterranean Sea. BMC Vet Res. 9(1):106. doi:10.1186/1746-6148-9-106.

Russell GC, Stewart JP, Haig DM. 2009. Malignant catarrhal fever: A review. Vet J. 179(3):324–335. doi:10.1016/j.tvjl.2007.11.007.

Saliki J, E C, Rotstein, Mclellan W a, Govett P, Harms C, Smolarek K a, Romero CH. 2006. A novel gammeherpesvirus associates with genital lesions in a blainville's beaked whale (Mesoplondon densirostris). J Wildl Dis. 42(1):142–148.

Santín Cerezales M, Benítez JD. 2011. Diagnosis of tuberculosis infection using interferon-γ-based assays. Enferm Infecc Microbiol Clin. 29(SUPPL. 1):26–33. doi:10.1016/S0213-005X(11)70015-5.

Sato H, Yoneda M, Honda T, Kai C. 2012a. Morbillivirus receptors and tropism: Multiple pathways for infection. Front Microbiol. 3(MAR). doi:10.3389/fmicb.2012.00075.

Sato H, Yoneda M, Honda T, Kai C. 2012b. Morbillivirus Receptors and Tropism: Multiple Pathways for Infection. Front Microbiol. 3(March):1–9.

doi:10.3389/fmicb.2012.00075.

Schneider-Schaulies S, Schneider-Schaulies J. 2009. Measles virus-induced immunosuppression. Curr Top Microbiol Immunol. 330:243–269. doi:10.1007/978-3-540-70617-5_12.

Schwartzberg PL, Mueller KL, Qi H, Cannons JL. 2009. SLAM receptors and SAP influence lymphocyte interactions, development and function. Nat Rev Immunol. 9(1):39–46. doi:10.1038/nri2456.

Di Sciara GN, Venturino MC, Zanardelli M, Bearzi G, Borsani FJ, Cavalloni B. 1993. Cetaceans in the central Mediterranean Sea: Distribution and sighting frequencies. Bolletino di Zool. 60(1):131–138. doi:10.1080/11250009309355800.

Seimon TA, Olson SH, Lee KJ, Rosen G, Ondzie A, Cameron K, Reed P, Anthony SJ, Joly DO, McAloose D, et al. 2015. Adenovirus and Herpesvirus Diversity in Free-Ranging Great Apes in the Sangha Region of the Republic of Congo. PLoS One. 10(3). doi:10.1371/journal.pone.0118543.

Sequeira M, Inácio A, Reiner F. Arrojamentos de mamíferos marinhos na costa continental portuguesa entre 1978 e 1988. Estudos de Biologia e Conservação da Natureza 7, SNPRCN, p. 48. 1992.

Shimizu Y, Ohishi K, Suzuki R, Tajima Y, Yamada T, Kakizoe Y, Bando T, Fujise Y, Taru H, Murayama T, et al. 2013a. Amino acid sequence variations of signaling lymphocyte activation molecule and mortality caused by morbillivirus infection in cetaceans. Microbiol Immunol. 57(9):624–32. doi:10.1111/1348-0421.12078.

Shimizu Y, Ohishi K, Suzuki R, Tajima Y, Yamada T, Kakizoe Y, Bando T, Fujise Y, Taru H, Murayama T, et al. 2013b. Amino acid sequence variations of signaling lymphocyte activation molecule and mortality caused by morbillivirus infection in cetaceans. Microbiol Immunol. 57(9):624–632. doi:10.1111/1348-0421.12078.

Sierra E, Díaz-Delgado J, Arbelo M, Andrada M, Sacchini S, Fernández A. 2015. HERPESVIRUS-ASSOCIATED GENITAL LESIONS IN A STRANDED STRIPED DOLPHIN (*STENELLA COERULEOALBA*) IN THE CANARY ISLANDS, SPAIN. J Wildl Dis. 51(3):696–702. doi:10.7589/2014-07-185.

Sierra E., Sanchez S, Saliki JT, Blas-Machado U, Arbelo M, Zucca D, Fernandez a. 2014. Retrospective Study of Etiologic Agents Associated with Nonsuppurative Meningoencephalitis in Stranded Cetaceans in the Canary Islands. J Clin Microbiol. 52(7):2390–2397. doi:10.1128/JCM.02906-13.

Sierra Eva, Zucca D, Arbelo M, García-Álvarez N, Andrada M, Déniz S, Fernández A. 2014. Fatal systemic morbillivirus infection in bottlenose dolphin, Canary Islands, Spain. Emerg Infect Dis. 20(2):269–271. doi:10.3201/eid2002.131463.

Sim RR, Norton TM, Bronson E, Allender MC, Stedman N, Childress AL, Wellehan JFX. 2015. Identification of a novel herpesvirus in captive Eastern box turtles (Terrapene carolina carolina). Vet Microbiol. 175(2–4):218–223. doi:10.1016/J.VETMIC.2014.11.029.

Sinclair C, Sinclair J, Zolman E, Martinez A, Balmer B, Barry K. 2015. NOAA Technical Memorandum NMFS-SEFSC-670 REMOTE BIOPSY SAMPLING FIELD PROCEDURES FOR CETACEANS USED DURING THE NATURAL RESOURCE DAMAGE ASSESSMENT OF THE MSC252 DEEPWATER HORIZON OIL SPILL. doi:10.7289/V5CC0XN0.

Smolarek Benson K a, Manire C a, Ewing RY, Saliki JT, Townsend FI, Ehlers B, Romero CH. 2006. Identification of novel alpha- and gammaherpesviruses from cutaneous and mucosal lesions of dolphins and whales. J Virol Methods. 136(1–2):261–6. doi:10.1016/j.jviromet.2006.03.033.

Song GY, Chung CS, Chaudry IH, Ayala A. 1999. What is the role of interleukin 10 in polymicrobial sepsis: Anti- inflammatory agent or immunosuppressant? Surgery. 126(2):378–383. doi:10.1016/S0039-6060(99)70180-7.

Soto S, Alba A, Ganges L, Vidal E, Raga JA, Alegre F, González B, Medina P, Zorrilla I, Martínez J, et al. 2011. Post-epizootic chronic dolphin morbillivirus infection in Mediterranean striped dolphins Stenella coeruleoalba. Dis Aquat Organ. 96(3):187–194. doi:10.3354/dao02387.

Soto S, González B, Willoughby K, Maley M, Olvera a., Kennedy S, Marco a., Domingo M. 2012. Systemic Herpesvirus and Morbillivirus Co-Infection in a Striped Dolphin (Stenella coeruleoalba). J Comp Pathol. 146:269–273. doi:10.1016/j.jcpa.2011.04.002.

Stephens N, Duignan PJ, Wang J, Bingham J, Finn H, Bejder L, Patterson IAP,

Holyoake C. 2014. Cetacean morbillivirus in coastal indo-pacific bottlenose dolphins, Western Australia. Emerg Infect Dis. 20(4):666–670. doi:10.3201/eid2004.131714.

Stone BM, Blyde DJ, Saliki JT, Morton JM. 2012. Morbillivirus infection in live stranded, injured, trapped, and captive cetaceans in southeastern Queensland and northern New South Wales, Australia. J Wildl Dis. 48(1):47–55. doi:10.7589/0090-3558-48.1.47.

Storch GA, Wang D. 2013. Diagnostic Virology. In: Knipe DM, Howley PM, editors. Field's virology. 6.ª ed. Filadelfia: Lippincott Williams & Wilkins.

De Swart RL, Ludlow M, De Witte L, Yanagi Y, Van Amerongen G, McQuaid S, Yüksel S, Geijtenbeek TBH, Duprex WP, Osterhaus ADME. 2007. Predominant infection of CD150+ lymphocytes and dendritic cells during measles virus infection of macaques. PLoS Pathog. 3(11):1771–1781. doi:10.1371/journal.ppat.0030178.

Tatsuo H, Ono N, Tanaka K, Yanagi Y. 2000. Slam (CDw150) is a cellular receptor for measles virus. Nature. 406(6798):893–897. doi:10.1038/35022579.

Tatsuo H, Ono N, Yanagi Y. 2001. Morbilliviruses Use Signaling Lymphocyte Activation Molecules (CD150) as Cellular Receptors. J Virol. 75(13):5842–5850. doi:10.1128/JVI.75.13.5842.

Taubenberger JK, Tsai M, Krafft a E, Lichy JH, Reid a H, Schulman FY, Lipscomb TP. 1996. Two morbilliviruses implicated in bottlenose dolphin epizootics. Emerg Infect Dis. 2(3):213–6. doi:10.3201/eid0203.960308.

Taubenberger JK, Tsai MM, Atkin TJ, Fanning TG, Krafft a E, Moeller RB, Kodsi SE, Mense MG, Lipscomb TP. 2000. Molecular genetic evidence of a novel morbillivirus in a long-finned pilot whale (Globicephalus melas). Emerg Infect Dis. 6(1):42–5. doi:10.3201/eid0601.000107.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22(22):4673–4680. doi:10.1093/nar/22.22.4673.

Tizard IR. 2009. Veterinary immunology : an introduction. 8th ed. St. Louis Mo.: Saunders.

Valsecchi E, Amos W, Raga JA, Podestà M, Sherwin W. 2004. The effects of inbreeding on mortality during a morbillivirus outbreak in the Mediterranean striped dolphin (Stenella coeruleoalba). Anim Conserv. 7(2):139–146. doi:10.1017/S1367943004001325.

VanDevanter DR, Warrener P, Bennett L, Schultz ER, Coulter S, Garber RL, Rose TM, Devanter DRVAN, Warrener P, Bennett L, et al. 1996. Detection and Analysis of Diverse Herpesviral Species by Consensus Primer PCR. J Clin Microbiol. 34(7):1666–1671.

De Veer MJ, Holko M, Frevel M, Walker E, Der S, Paranjape JM, Silverman RH, Williams BRG. 2001. Functional classification of interferon-stimulated genes identified using microarrays. John Wiley & Sons, Ltd.

Veillette A, Dong Z, Latour S. 2007. Consequence of the SLAM-SAP Signaling Pathway in Innate-like and Conventional Lymphocytes. Immunity. 27(5):698–710. doi:10.1016/j.immuni.2007.11.005.

Veillette A, Latour S. 2003. The SLAM family of immune-cell receptors. Curr Opin Immunol. 15(3):277–285. doi:10.1016/S0952-7915(03)00041-4.

Vingada J, Marçalo A, Ferreira M, Eira C, Henriques A, Miodonsky J, Oliveira N, Marujo D, Almeida A, Barros N, et al. 2012. Capítulo I: Interações entre as espécies-alvo e as pescas. Anexo ao relatório intercalar do projecto LIFE MarPro NAT/PT/00038.

Vingada, J., Ferreira, M., Marçalo, A., Santos, J., Araújo, H., Oliveira, I., Monteiro, S., Nicolau, L., Gomes, P., Tavares, C. and Eira, C. (2011). SAFESEA - Manual de Apoio para a Promoção de uma Pesca Mais Sustentável e de um Mar Seguro para Cetáceos. Programa EEAGrants - EEA Financial Mechanism 2004-2009 (Projecto 0039), Braga. 114 pp. In Portuguese.

Visser IKG, Kumarev VP, Örvell C, de Vries P, Broeders HWJ, van de Bildt MWG, Groen J, Teppema JS, Burger MC, UytdeHaag FGCM, et al. 1990. Comparison of two morbilliviruses isolated from seals during outbreaks of distemper in North West Europe and Siberia. Arch Virol. 111(3–4):149–164. doi:10.1007/BF01311050.

de Vries RD, McQuaid S, van Amerongen G, Yüksel S, Verburgh RJ, Osterhaus ADME, Duprex WP, de Swart RL. 2012. Measles Immune Suppression: Lessons from the Macaque Model. PLoS Pathog. 8(8). doi:10.1371/journal.ppat.1002885.

de Vries RD, de Swart RL. 2014. Measles Immune Suppression: Functional Impairment or Numbers Game? PLoS Pathog. 10(12). doi:10.1371/journal.ppat.1004482.

Wade PR. 1998. CALCULATING LIMITS TO THE ALLOWABLE HUMAN-CAUSED MORTALITY OF CETACEANS AND PINNIPEDS. Mar Mammal Sci. 14(1):1–37. doi:10.1111/j.1748-7692.1998.tb00688.x.

Watanabe A, Yoneda M, Ikeda F, Terao-Muto Y, Sato H, Kai C. 2010. CD147/EMMPRIN Acts as a Functional Entry Receptor for Measles Virus on Epithelial Cells. J Virol. 84(9):4183–4193. doi:10.1128/jvi.02168-09.

Waterhouse AM, Procter JB, Martin DM a, Clamp M, Barton GJ. 2009. Jalview Version 2-A multiple sequence alignment editor and analysis workbench. Bioinformatics. 25(9):1189–1191. doi:10.1093/bioinformatics/btp033.

Wellehan JFX, Jarchow JL, Reggiardo C, Jacobson ER. 2003. A novel herpesvirus associated with hepatic necrosis in a San Esteban chuckwalla, Sauromalus varius. J Herpetol Med Surg. 13(3):15–19.

West KL, Sanchez S, Rotstein D, Robertson KM, Dennison S, Levine G, Davis N, Schofield D, Potter CW, Jensen B. 2012. A Longman's beaked whale (*Indopacetus pacificus*) strands in Maui, Hawaii, with first case of morbillivirus in the central Pacific. Mar Mammal Sci. 29(October):n/a-n/a. doi:10.1111/j.1748-7692.2012.00616.x.

Wild TF, Malvoisin E, Buckland R. 1991. Measles virus: Both the haemagglutinin and fusion glycoproteins are required for fusion. J Gen Virol. 72(2):439–442. doi:10.1099/0022-1317-72-2-439.

Wilkinson DM. 1996. National Contingency Plan for Response to Unusual Marine Mammal Mortality Events (NMFS-OPR-9).

Won EJ, Choi JH, Cho YN, Jin HM, Kee HJ, Park YW, Kwon YS, Kee SJ. 2017.

Biomarkers for discrimination between latent tuberculosis infection and active tuberculosis disease. J Infect. 74(3):281–293. doi:10.1016/j.jinf.2016.11.010.

Woo PCY, Lau SKP, Wong BHL, Fan RYY, Wong AYP, Zhang AJX, Wu Y, Choi GKY, Li KSM, Hui J, et al. 2012. Feline morbillivirus, a previously undescribed paramyxovirus associated with tubulointerstitial nephritis in domestic cats. Proc Natl Acad Sci U S A. 109(14):5435–5440. doi:10.1073/pnas.1119972109.

Würsig, B. & Pearson, H.C. (2015) Dolphin societies: Structure and function. In Herzing, D.L. & Johnson, C. M. (Eds.), Dolphin Communication and Cognition: Past, Present and Future. Cambrige: MIT Press (pp. 77-105)

Yu X, Cheng Y, Shi B, Qian F, Wang F, Liu X, Yang H, Xu Q, Qi T, Zha L, et al. 2008. Measles Virus Infection in Adults Induces Production of IL-10 and Is Associated with Increased CD4 + CD25 + Regulatory T Cells . J Immunol. 181(10):7356–7366. doi:10.4049/jimmunol.181.10.7356.

Zhao S, Fernald RD. Comprehensive Algorithm for Quantitative Real-Time Polymerase Chain Reaction. doi:10.1089/cmb.2005.12.1047.