ULPGC
Universidad de
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Escuela de
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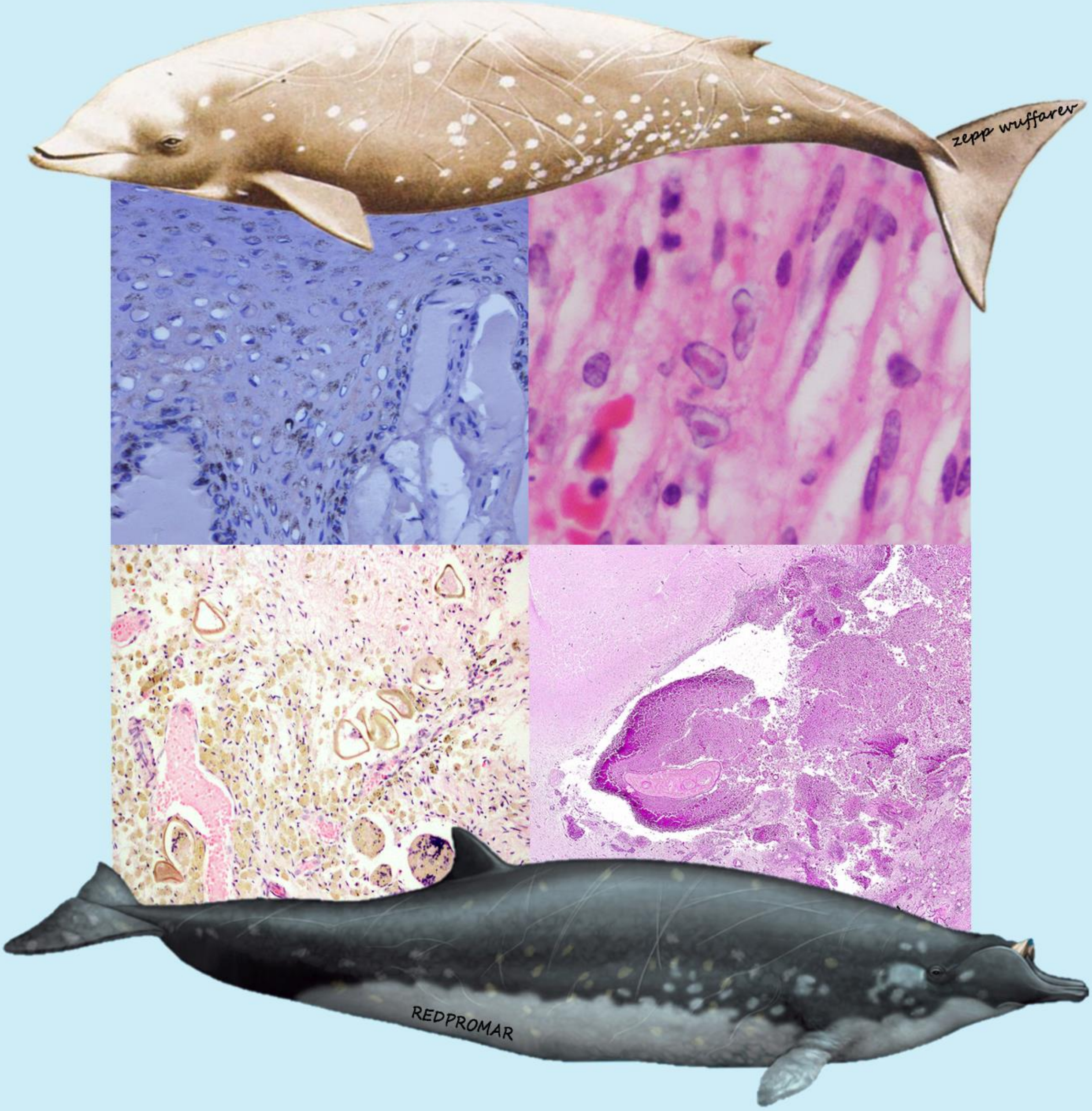
TESIS DOCTORAL

EVALUACIÓN SANITARIA DE LOS ZIFIOS EN CANARIAS



IDAIRA DEL CARMEN FELIPE JIMÉNEZ

DOCTORADO EN SANIDAD ANIMAL Y SEGURIDAD ALIMENTARIA
LAS PALMAS DE GRAN CANARIA
SEPTIEMBRE 2023

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Tesis presentada por: **D ª. Idaira del Carmen Felipe Jiménez.**

Dirigida por: **Dr. Antonio Fernández Rodríguez** y por la **Dra. Eva Sierra Pulpillo.**

El Director:	La Directora:	La Doctoranda:
(Firma)	(Firma)	(Firma)

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D. ANTONIO FERNÁNDEZ RODRÍGUEZ, COORDINADOR DEL PROGRAMA DE DOCTORADO DE SANIDAD ANIMAL Y SEGURIDAD ALIMENTARIA DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA,

INFORMA,

Que la Comisión Académica del Programa de Doctorado, en su sesión de fecha / / tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada "**EVALUACIÓN SANITARIA DE LOS ZIFIOS EN CANARIAS**" presentada por la doctoranda Dª **Idaira del Carmen Felipe Jiménez** y dirigida por el Catedrático **Antonio Fernández Rodríguez** y la Doctora **Eva Sierra Pulpillo**.

Y para que así conste, y a efectos de lo previsto en el Artº 11 del Reglamento de Estudios de Doctorado (BOULPGC 7/10/2016) de la Universidad de Las Palmas de Gran Canaria, firmo la presente en Las Palmas de Gran Canaria, a de del dos mil veintitrés.

A mis padres

A Negrito, Siberia, Ranita y Pi.

"Un solo sueño es más poderoso
que mil realidades."

J.R.R. TOLKIEN

INDICE:

> “Cette tête est très-pesante, très-dure et complétement pétrifiée en calcaire. J'appliquerai au genre dont elle devient le premier type, le nom de ZIPHIUS, employé par quelques auteurs du moyen âge (voyez Gesner, I, p. 209) pour un cétacé qu’ils n’ont point déterminé, et je nommerai cette espèce *ziphius cavirostris*".*

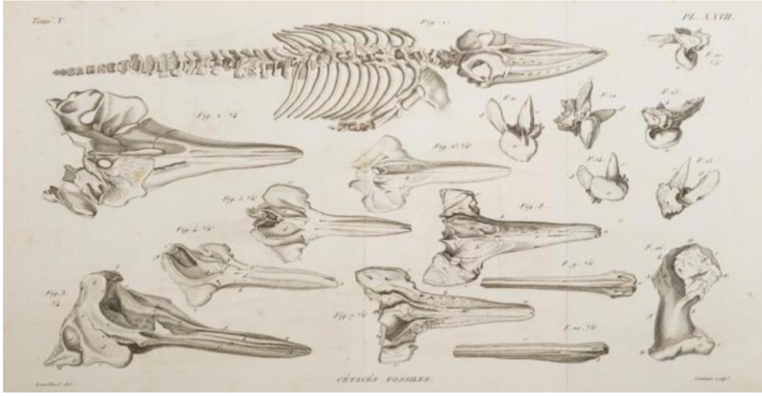
George Cuvier

Recherches sur les ossements fossiles, où l'on rétablit les caractères de plusieurs animaux dont les révolutions du globe ont détruit les espéces; Par M. le Bon. G. Cuvier, NOUVELLE EDITION, Tome cinquème, 1re. partie, (Paris, G. Dufour et E. D'Ocagne, p. 352, Libraires, quai Voltaire, Nº. 13. Et a Amsterdam, chez les mémes. 1823.

***

Traducción:

"Esta cabeza es muy pesada, muy dura y completamente petrificada en piedra caliza. Aplicaré al género que se convierte en el primero de su tipo, el nombre de ZIPHIUS, empleado por algunos autores de la Edad Media (ver Gesner, I, p. 209) para un cetáceo que no han determinado, y nombraré a esta especie *ziphius cavirostris*").



***Nota del autor:** Primera descripción de la especie zifio de Cuvier (*Ziphius cavirostris*), realizada por George Cuvier en 1823 en su “*Recherches sur les ossements fossiles, où l'on rétablit les caractères de plusieurs animaux dont les révolutions du globe ont détruit les espéces*”, a partir de un cráneo encontrado en 1804 por el ingeniero civil Raymond Gorsse en las costas de la Provenza en Francia (Figura 3, Plano XXVII) (Cuvier, 1823).

1. ABREVIATURAS

Zifios de Blainville (*Mesoplodon densirostris*). FOTOGRAFÍA: J. Alcazar / Universidad de La Laguna (ULL)

1. ABREVIATURAS

ADN: Ácido desoxirribonucleico.

ARN: Ácido ribonucleico.

AT: Testículo anterior.

BWCV: *Circovirus de los zifios.*

BWMV: *Morbillivirus de los zifios.*

CeMBV: *Morbillivirus de los cetáceos.*

- **CeMV-1:** Linajes del hemisferio norte.
- **CeMV-2:** Linajes del hemisferio sur.

CDC: Centers for Disease Control and Prevention.

COVID-19: Coronavirus del 2019.

DMV: *Morbillivirus de los delfines*.

EP: poro excretor

EE.UU.: Estados Unidos.

F: Proteína de fusión.

GP: poro genital.

H: Proteína de la hemaglutinina.

HV: Herpesvirus.

- **αHV:** *Alphaherpesvirus*.
- **βHV:** *Betaherpesvirus*.
- **УHV:** *Gammaherpesvirus*.

IC: Ciego intestinal.

IUSA: Instituto de Sanidad Animal y Seguridad Alimentaria.

L: Proteína de ARN polimerasa dependiente de ARN.

m: metros.

M: Proteína matriz.

N: Nucleoproteína.

NH: Proteína de la hemaglutinina-neuraminidasa.

O: Ovario.

OS: Ventosa oral.

P: Fosfoproteína.

P: Faringe.

PMV: *Morbillivirus de las marsopas.*

PT: Testículo posterior.

PWMV: *Morbillivirus de los calderones*.

SARS-Cov-2: Coronavirus de tipo 2 del Síndrome respiratorio agudo severo.

SECAC: Sociedad para el Estudio de los Cetáceos en el Archipiélago Canario.

Sp.: Especie.

Spp.: Especies.

U: Útero.

VG: Glándula vitelógenas o vitellaria.

VS: Acetábulo.

2. INTRODUCCIÓN

Zifio de Cuvier (*Ziphius cavirostris*). FOTOGRAFÍA: Vidal Martín / Sociedad para el Estudio de los Cetáceos en el Archipiélago Canario (SECAC)

2. INTRODUCCIÓN

2.1. IMPORTANCIA DEL CONCEPTO "ONE HEALTH"

Históricamente y desde tiempos ancestrales, las enfermedades infecciosas han supuesto una amenaza para los seres vivos de este planeta, sin embargo, también han constituido un elemento para el control de la población y un impulso en la evolución de las especies.

Con el origen de la agricultura y la domesticación de los animales, se produce un proceso de convivencia entre los microorganismos, los animales salvajes, domésticos y el ser humano; pudiendo ser esta interacción pacífica o nociva. Prueba de esto último son las pandemias y epidemias ocurridas a lo largo de la historia de la humanidad, siendo la reciente pandemia de la Enfermedad por coronavirus del 2019 (COVID-19), causada por el virus del Coronavirus de tipo 2 del Síndrome respiratorio agudo severo (SARS-Cov-2) y de posible origen animal, la última crisis sanitaria a nivel mundial a la que se nos hemos tenido que enfrentar. Es por ello por lo que el concepto One Health ("Una sola salud") ha cobrado especial relevancia como estrategia internacional con el fin de promover la colaboración interdisciplinar en el cuidado de la salud de las personas, los animales y el medio ambiente (WOAH: The Wold Organisation for Animal Health, 2003).

En este sentido, la sanidad de la fauna silvestre se encuentra estrechamente vinculada a la de los animales domésticos, el hábitat e incluso a la de los seres humanos; ya que los animales salvajes contribuyen al equilibrio de los ecosistemas en los que viven. Así mismo, los Programas de Vigilancia Epidemiológica de Fauna Silvestre, llevan a cabo el seguimiento del estado sanitario de las especies silvestres con objeto de detectar, vigilar y/o controlar la aparición de enfermedades infecciosas de tipo zoonóticas, zooantroponóticas, emergentes y re-emergentes. De esta labor se encarga el Instituto de Sanidad Animal y Seguridad Alimentaria (IUSA) de la Universidad de Las Palmas de Gran Canaria, mediante proyectos enmarcados dentro de la Red de Varamientos de Cetáceos de Canarias y la Red de Vigilancia de Fauna Silvestre de Canarias, en colaboración con otras instituciones tanto de carácter gubernamental (Gobierno de Canarias y Cabildos Insulares) como no gubernamental de carácter científico (Canarias Conservación y la Sociedad para el Estudio de los Cetáceos en el Archipiélago Canario (SECAC)), así como campañas de investigación realizadas por otros grupos de investigación de cetáceos del archipiélago.

Sobre la base de lo anteriormente comentado, concretamente en el medio marino, los cetáceos han de hacer frente a diversas adversidades tanto de origen natural como antrópico, que pueden causar el desarrollo de patologías de curso agudo, sub- agudo y/o crónico e incluso provocar la muerte de los mismos. Es por ello que los cetáceos se convierten en bioindicadores del estado sanitario de los ecosistemas marinos, lo que permite evaluar el grado de degradación o conservación que las actividades humanas generan en los mismos; así como controlar y vigilar la aparición de enfermedades emergentes y reemergentes, que podrían dar lugar al surgimiento de importantes epizootias a nivel mundial tanto para especies marinas como terrestres; o zoonosis que podrían afectar al ser humano.

2.2. SITUACIÓN GEOGRÁFICA Y CARACTERÍSTICAS GEOLÓGICAS Y OCÉANOGRÁFICAS DE LAS ISLAS CANARIAS

Las Islas Canarias forman parte de la región de la Macaronesia y se encuentran situadas en el límite centro oriental del Océano Atlántico y al noroeste de África, cerca de las costas del sur de Marruecos y del norte del Sáhara Occidental entre las coordenadas 27° 37' y 29° 25' de latitud norte y 13° 20' y 18° 10' de longitud oeste. El archipiélago canario se encuentra conformado por un total de ocho islas, tres orientales (Fuerteventura, Lanzarote y La Graciosa), dos centrales (Gran Canaria y Tenerife) y tres occidentales (La Gomera, La Palma y el Hierro), además de algunos islotes (Montaña Clara, Alegranza, Roque del Este, Roque del Oeste, Isla de Lobos) y numerosos roques (Salmor, Fasnia, Bonanza, Garachico, Anaga, Gando y el Farallón de Sardina) (Gobierno de Canarias, 2011).

Son islas de naturaleza volcánica, resultado de un largo proceso geológico de millones de años, donde tras sucesivas erupciones volcánicas y aglomeraciones de capas de lava les han permitido sobrepasar la superficie del mar y dar lugar a las mismas. Las islas Canarias emergen desde una profundidad de 200 metros (m) la cual aumenta hasta los 1200 m hacia al oeste de Lanzarote y Fuerteventura, así como también alcanza los 4000 m de profundidad en las islas más occidentales (Agudo-Bravo & Mangas, 2015; Mangas y cols., 2015) **(Figura 1)**. Además, hay que destacar que carecen de plataforma continental y se caracterizan por sus fondos abruptos formados por acantilados submarinos, cuevas, túneles y cornisas. La plataforma insular se extiende hasta los 100-200 metros de profundidad y, aunque es accidentada y estrecha, es el lugar donde coexisten diversas especies litorales (Agudo-Bravo & Mangas, 2015). En cuanto a sus características oceanográficas, la corriente fría de Canarias, con origen en la Corriente del Golfo, le confiere a sus aguas unas temperaturas templadas y cálidas (17-18ºC en invierno y 22-23ºC en verano) que, junto a los fenómenos de afloramiento que se producen en la costa africana y gracias a la acción de los vientos alisios, les proporciona a las mismas un importante flujo de microorganismos y nutrientes procedentes de las regiones norte y occidental de océano Atlántico, y del Mar Mediterráneo. En cuanto a la salinidad de las aguas, éstas suelen presentar valores estables (Valdés & Déniz-González, 2015). En definitiva, todos estos factores oceanográficos, físico-químicos, así como las características morfológicas del fondo marino le confieren a nuestras islas una amplia variedad y riqueza de especies marinas procedentes del Caribe, de la costa africana, del Atlántico nororiental y occidental y del mar Mediterráneo; así como una gran cantidad de especies endémicas marinas.

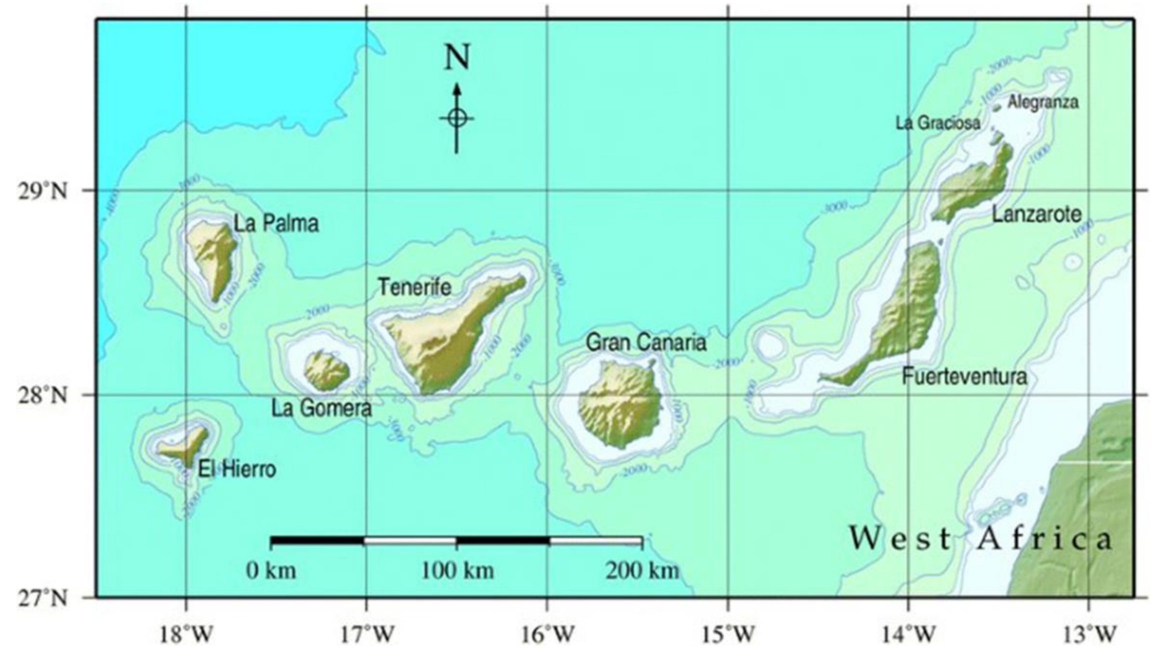
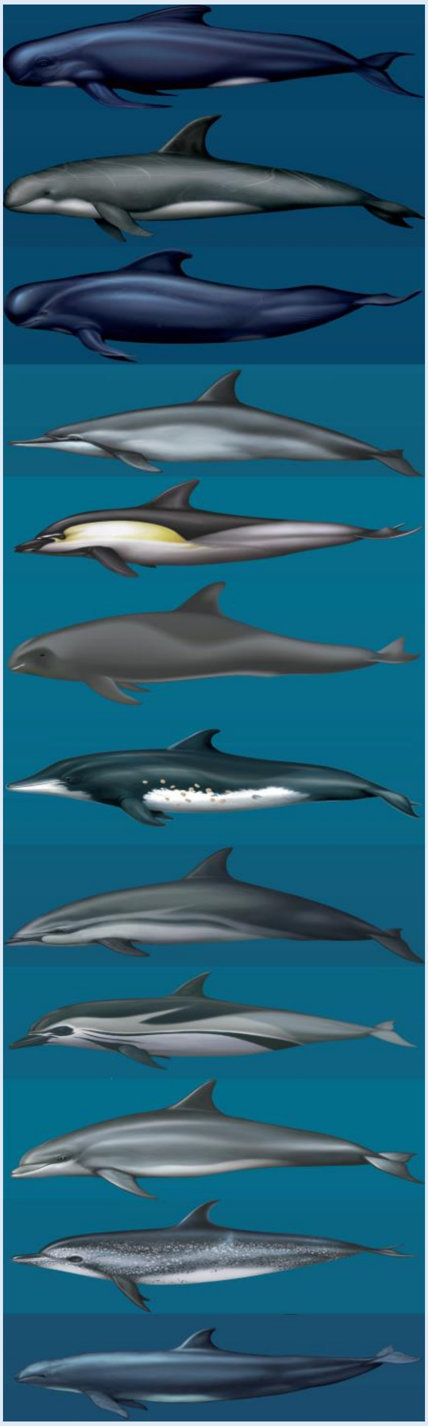


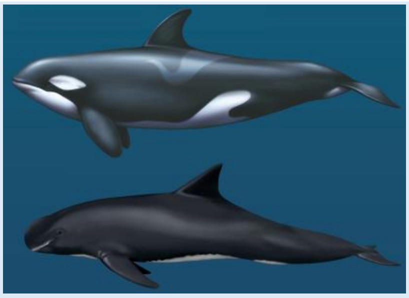
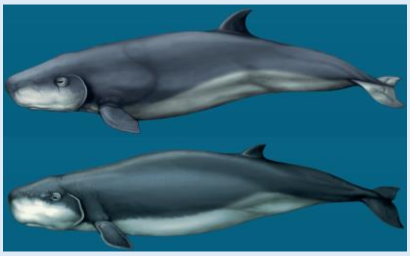
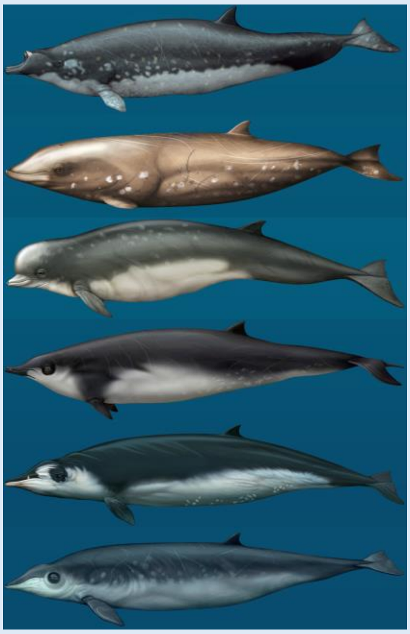
Figura 1: Mapa batimétrico de las Islas Canarias, detalle de las profundidades marinas del archipiélago canario. Ilustración correspondiente al artículo: *An Overview of Geodetic Volcano Research in the Canary Islands* por (Fernández y cols., 2015).

Las aguas de las Islas Canarias presentan una gran biodiversidad dentro de la cual se han llegado a identificar 31 especies diferentes de cetáceos, siendo 24 especies de odontocetos y 7 de misticetos (Sociedad para el Estudio de cetáceos en el Archipiélago Canario (SECAC), 2022), dichas especies se especifican en la **(Tabla 1 A y B).**

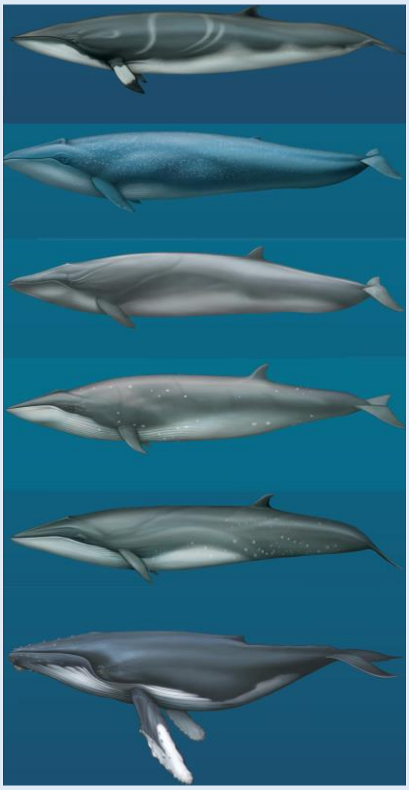
Tabla 1: Registro actualizado del 2022 de las especies de cetáceos observadas en las Islas Canarias. **A)** Ejemplares correspondientes al Suborden *Odontoceti*. **B)** Individuos correspondientes al Suborden *Mysticeti*. *Imágenes obtenidas de la página web de (REPROMAR: Red de Observadores del Medio Marino en Canarias, 2023), pertenecientes a Ilustraciones Oceanográfica. La imagen de la especie *Peponocephala electra* aquí reproducida pertenece a la siguiente fuente (The Society for The Marine Mammalogy, 2023).

(A)

Suborden *Odontoceti*		
Familia *Delphinidae*	• **Calderón común** (*Globicephala melas*) • **Calderón gris** (*Grampus griseus*) • **Calderón tropical** (*Globicephala macrorhynchus*) • **Delfín acróbata de hocico largo** (*Stenella longirostris*) • **Delfín común** (*Delphinus delphis*) • **Delfín de cabeza de melón** (*Peponocephala electra*) • **Delfín de dientes rugosos** (*Steno bredanensis*) • **Delfín de Fraser** (*Lagenodelphis hosei*) • **Delfín listado** (*Stenella coeruleoalba*) • **Delfín mular** (*Tursiops truncatus*) • **Delfín moteado del Atlántico** (*Stenella frontalis*) • **Falsa orca** (*Pseudorca crassidens*)	

Suborden *Odontoceti* *(continuación)		
Familia *Delphinidae* (*continuación)	- **Orca** (*Orcinus orca*) - **Orca pigmea** (*Feresa attenuata*)	
Familia *Kogiidae*	- **Cachalote enano** (*Kogia sima*) - **Cachalote pigmeo** (*Kogia breviceps*)	
Familia *Physeteridae*	- **Cachalote** (*Physeter macrocephalus*)	
Familia *Phocoenidae*	- **Marsopa común** (*Phocoena phocoena*)	
Familia *Ziphiidae*	- **Zifio de Blainville** (*Mesoplodon densirostris*) - **Zifio de Cuvier** (*Ziphius cavirostris*) - **Zifio Calderón Boreal** (*Hyperoodon ampullatus*) - **Zifio de Gervais** (*Mesoplodon europaeus*) - **Zifio de Sowerby** (*Mesoplodon bidens*) - **Zifio de True** (*Mesoplodon mirus*)	

(B)

Suborden *Mysticeti*		
Familia *Balaenidae*	• **Ballena franca** (*Eubalaena glacialis*)	
Familia *Balenopteridae*	• **Rorcual aliblanco** (*Balaenoptera acutorostrata*) • **Rorcual azul** (*Balaenoptera musculus*) • **Rorcual común** (*Balaenoptera physalus*) • **Rorcual norteño** (*Balaenoptera borealis*) • **Rorcual tropical** (*Balaenoptera edeni*) • **Yubarta** (*Megaptera novaeangliae*)	

2.3. LA FAMILIA *ZIPHIIDAE* Y SUS AMENAZAS EN EL MEDIO MARINO

La familia *Ziphiidae*, clasificada dentro del Parvorden *Odontoceti,* se encuentra actualmente compuesta por 23 especies agrupadas en 6 géneros (Carroll y cols., 2021; MacLeod, 2018) **(Figura 2)**. Esta categoría taxonómica fue establecida por John Edward Gray en el año 1865 en su catálogo de focas y ballenas del Museo Británico (Mead, 2009a).

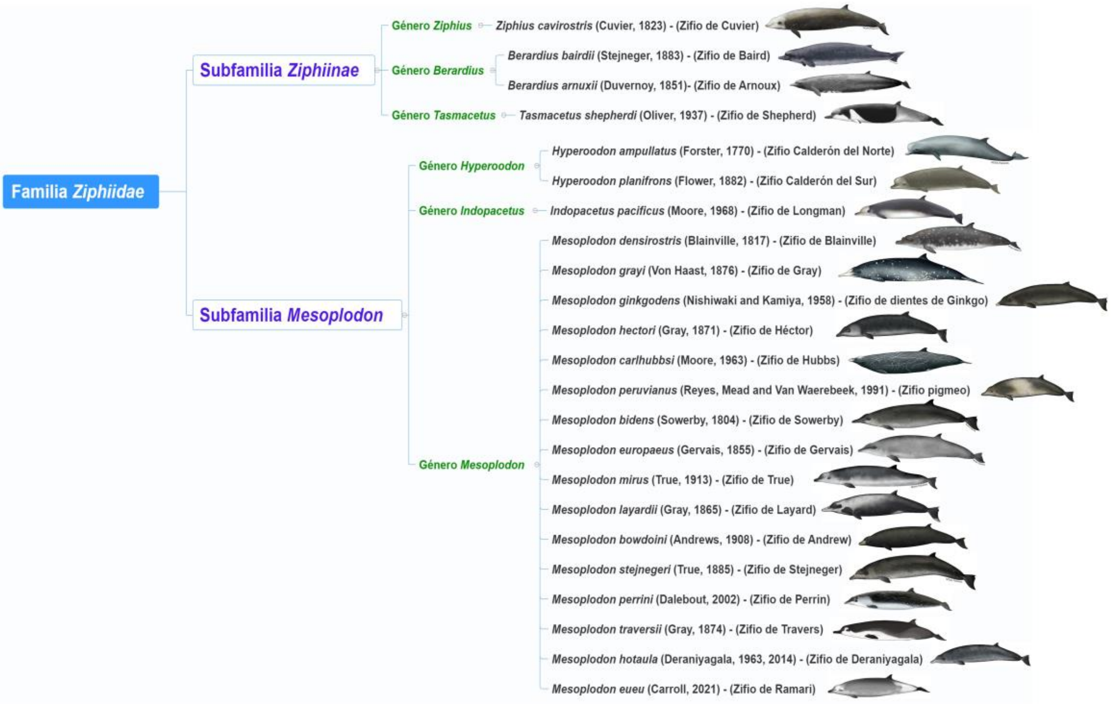


Figura 2: Árbol taxonómico de la Familia *Ziphiidae*. Diseñado mediante los programas informáticos (Adobe Photoshop CS6, 2017; Mindonmap.com, 2023). Las imágenes de las distintas especies de zifios se han obtenido de las siguientes fuentes: Zifio de Cuvier, zifio de Baird, zifio calderón del norte, zifio de Blainville, zifio de Sowerby, zifio de Gervais, zifio de True y zifio de Stejnerger (National Oceanic and Atmospheric Administration (NOAA Fisheries), 2020). Zifio de Shepherd (Mead, 2009b). Zifio Calderón del Norte y zifio de Longman (Moors-Murphy, 2018; Pitman, 2018). Zifio de Arnoux (iNaturalist Ecuador, 2023). Zifio de Gray (Westbury y cols., 2021). Zifio de dientes de Ginkgo, zifio de Héctor, zifio pigmeo y zifio de Deraniyagala (Marine Life Project, 2020). Zifio de Hubbs, zifio de Layard y zifio de Andrew (Martin Camm, 2011; Yumiko Wakisaka, 2014b, 2014a). Zifio de Perrin (Folkens, 2023). Zifio de Travers (Thompson y cols., 2012) y zifio de Ramari (Carroll y cols., 2021).

Los zifios se encuentran ampliamente distribuidos por todos los océanos del planeta; se tratan de animales con hábitos pelágicos y de buceo profundo, pudiendo permanecer largos períodos de tiempo sumergidos a gran profundidad (MacLeod, 2018; Schorr y cols., 2014). Estos odontocetos se caracterizan por tener un comportamiento muy evasivo con el ser humano, por esta razón son de gran interés dentro del mundo científico debido al gran desconocimiento existente sobre ellos, pues en muchos casos los datos de los que se disponen provienen en su mayor medida de animales varados. Una de las especies de zifio de las que mayor conocimiento en ecología y biología se dispone es el zifio calderón del Norte (*Hyperoodon ampullatus*), debido a que en pleno siglo XIX fueron objeto de captura por la industria ballenera y como resultado de ello la población fue sobreexplotada, siendo en 1970 protegidas por la Comisión Ballenera Internacional para su conservación (Mead, 2009a).

Los miembros de la familia *Ziphiidae* presentan diversos tamaños dependiendo de la especie, pueden llegar a medir entre 3 y 13 metros, siendo el zifio enano (*Mesoplodon peruvianus*) el ejemplar que menor talla presenta y el zifio de Baird (*Berardius bairdi*) el de mayor tamaño y peso. Por lo general, las hembras suelen presentar un mayor tamaño que los machos (Mead, 2009a).

Los zifios se caracterizan por un cuerpo robusto, hidrodinámico y morfológicamente en forma de cigarro, en el que externamente presenta marcas y cicatrices producidas por la interacción entre miembros del mismo grupo (Heyning & Mead, 2009) **(Figura 3)**. También se han descrito algunas particularidades en comparación con otras especies de odontocetos como: la presencia de un rostro bastante pronunciado y la disposición de las aletas, en donde la escotadura caudal es casi inexistente en la aleta caudal y donde tanto las pectorales como la dorsal suelen ser de tamaño pequeño (si bien ésta última se caracteriza por ubicarse en una posición más caudal en relación al eje central del cuerpo). Otra peculiaridad relevante de este grupo de animales es la presencia de dos surcos en forma de V en la porción ventral y media de la región cefálica, denominados surcos gulares (Heyning & Mead, 2009).

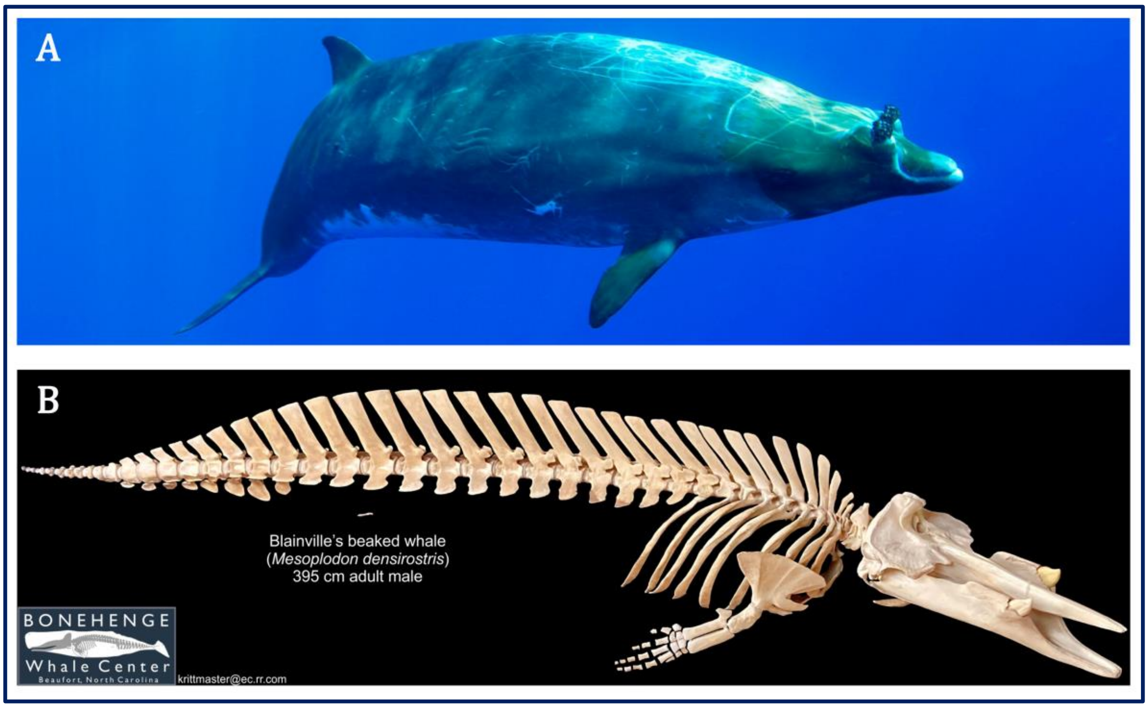


Figura 3: Panel de imágenes realizado con el programa (Adobe Photoshop CS6, 2017), detalle la morfología del cuerpo de un zifio de Blainville (*Mesoplodon densisrostris*). Imágenes correspondientes a los siguientes autores: **A)** Ejemplar de macho adulto de zifio de Blainville (Lucas, 2020). **B)** Esqueleto completo de un macho adulto de zifio de Blainville varado en Ocracoke en febrero de 2015 (Rittmaster y cols., 2015).

Un distintivo de dimorfismo sexual para poder identificar el sexo y la edad, es la presencia de un par de dientes funcionales en la mandíbula que sobresalen de las encías, como ocurre en el caso de los machos de algunas especies **(Figura 4)**. La disposición de la hemimandíbula y los dientes permiten en muchos casos identificar la especie de zifio, como en el caso del zifio de Shepherd (*Tasmacetus shepherdii*), que conserva el mayor número de dientes en ambas hemimandíbulas (Mead, 2009a). Además, de manera evolutiva, la pérdida de piezas dentales se encuentra relacionada con la forma de alimentación mediante succión, que gracias a los surcos gulares les permiten expandir la garganta. La dieta de estos animales consiste principalmente en peces, crustáceos mesopelágicos y calamares pelágicos; habiéndose identificado en los estómagos de zifios del Océano Atlántico algunos ejemplares correspondientes a las Familias *Histioteuthidae, Enoploteuthidae, Gonatidae, Octopoteuthidae, Brachioteuthidae y Chiroteuthidae* (Canarias Conservación., 2018).

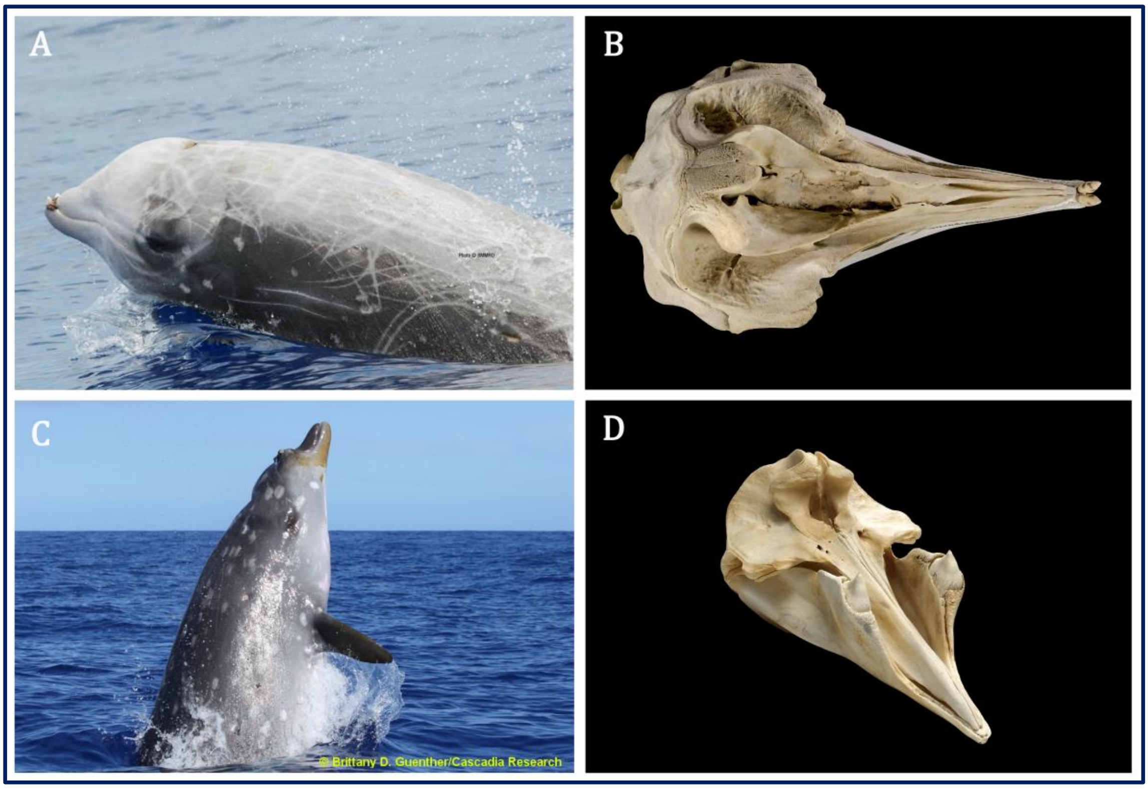


Figura 4: Panel de imágenes realizado con el programa (Adobe Photoshop CS6, 2017). Detalle del dimorfismo sexual que presentan algunas especies de machos de zifios, en el que se observa la presencia de los dientes funcionales que sobresalen de las encías. Imágenes obtenidas y correspondientes a los siguientes autores: **A)** Detalle de la región cefálica y dientes funcionales de un ejemplar de macho de zifio de Cuvier (*Ziphius cavirostris*) (Bahamas Marine Mammal Res. Org. & Berry, 2019). **B)** Cráneo de un ejemplar de zifio de Cuvier hallado cerca de la salida de la laguna Ohuia, en Nueva Zelanda en el año 1996, detalle de la presencia de un par de dientes en la porción rostral de la mandíbula (van Helden et al., 1996). **C)** Primer plano de la cabeza de un macho adulto de zifio de Blainville localizado frente a la isla de Hawái en el año 2019 (Guenther & Cascadia Research, 2019). **D)** Cráneo de un ejemplar de zifio de Blainville hallado en la playa de Tongoia, en Nueva Zelanda en el año 1998, detalle de la presencia de dientes que sobresalen de ambas hemimandíbulas (Rook & van Helden, 1998).

Finalmente hay que resaltar que en las Islas Canarias se ha registrado la presencia de, al menos, 6 especies de tres géneros diferentes consideradas residentes como son: el zifio de Cuvier (*Ziphius cavirostris*), el zifio de Blainville (*Mesoplodon densirostris*), el zifio de True (*Mesoplodon mirus*), el zifio de Sowerby (*Mesoplodon bidens*), el zifio de Gervais (*Mesoplodon europaeus*) y el zifio calderón boreal (*Hyperoodon ampullatus*) (Canarias Conservación., 2018) **(Figura 5)**.

Figura 5: Panel de imágenes realizado con el programa (Adobe Photoshop CS6, 2017), en donde se detallan las seis especies de zifios registradas y observadas en las Islas Canarias. Imágenes obtenidas y correspondientes a los siguientes autores: zifio de Cuvier (*Ziphius cavirostris*) (Aguilar, 2020), zifio de Blainville (*Mesoplodon densirostris*) (Lucas, 2020), zifio de True (*Mesoplodon mirus*) (Horsfall, 2018), zifio de Sowerby (*Mesoplodon bidens*) (Verdaat, 2015), zifio de Gervais (*Mesoplodon europaeus*) (Patteson y cols., 2019) y el zifio calderón boreal (*Hyperoodon ampullatus*) (EcuRed, 2012).

En el medio marino, la familia *Ziphiidae* está sometida a diversas amenazas de origen antrópico como pueden ser: los enredos en artes de pesca; la ingestión de basuras marinas y la colisión con embarcaciones (Puig-Lozano y cols., 2020a; Puig-Lozano y cols., 2020b; Sierra y cols., 2014). También se han visto involucrados en los varamientos masivos asociados al uso de sónares de media frecuencia utilizados en maniobras militares (Fernández y cols. 2012; Bernaldo De Quirós y cols., 2019; Fernández y cols., 2005; Frantzis, 1998; Simmonds & Lopez-Jurado, 1991). Asimismo, en esta familia de odontocetos se han descrito patologías de origen natural causadas por: virus, como el *morbillivirus de los cetáceos* (CeMBV), herpesvirus (HV) y más recientemente el circovirus de los zifios (BWCV))

(Arbelo y cols., 2012, 2010; Clifton y cols., 2023; Landrau-Giovannetti y cols., 2020; West y cols., 2013), parásitos, responsable de la arteritis verminosa por *Crassicauda spp*. (Díaz-Delgado y cols., 2016), bacterias, produciendo brucelosis en tres zifios de Sowerby (Davison cols., 2021) y septicemia por *Flavobacterium ceti* (Vela y cols., 2007), *Citrobacter freundii* (Fernández y cols., 2011) y *Morganella morganii* en la especie zifio de Cuvier (Romani-Cremaschi y cols., 2023).

Finalmente, y atendiendo a la necesidad de conocer y realizar una vigilancia y control de la aparición de posibles enfermedades emergentes y reemergentes entre los ejemplares de la familia *Ziphiidae* varados en las Islas Canarias, esta tesis doctoral se ha centrado principalmente en aquéllas patologías de origen natural vírico siendo el virus del *morbillivirus de los cetáceos* y el herpesvirus los que presentan una mayor importancia debido a que su virulencia cursa con altas morbilidades y mortandades en los varamientos de los cetáceos. Además, como consecuencia de la infección de estos virus en los zifios de estudio también se ha realizado una detección de posibles agentes patógenos secundarios oportunistas. En la presente tesis doctoral se describe un caso positivo a herpesvirus con la presencia de una encefalitis piogranulomatosa causada por *Nasitrema delphini*.

2.4. PATOLOGÍAS DE ORIGEN NATURAL VIRÍCO

2.4.1. HERPESVIRUS Y CASOS DETECTADOS EN ZIFIOS

Los virus pertenecientes al orden Herpesvirales se encuentran ampliamente distribuidos por todos los ecosistemas del planeta afectando a una gran variedad de especies de animales como: insectos, moluscos, peces, anfibios, reptiles, aves y mamíferos. Cada familia de herpesvirus se ha adaptado de manera específica a sus huéspedes, probablemente debido a una coevolución virus-hospedador (especie) producida a lo largo del tiempo (Carrasco & Almendral del Río, 2006; MacLachlan & Dubovi, 2011).

Son virus que se encuentran estructurados por una envoltura lipídica que contiene una membrana con glicoproteínas, un tegumento con proteínas víricas, una cápside de estructura icosaédrica y un núcleo cuyo interior presenta un genoma de una sola cadena doble de ácido desoxirribonucleico (ADN) lineal **(figura 6 A y B)** (MacLachlan & Dubovi., 2011)**.** Además, se caracterizan porque durante la fase de infección pueden utilizar las siguientes estrategias: manipulación del ciclo celular, actividad oncogénica, y evasión de la respuesta inmune mediante la interferencia. Además, hay que destacar su capacidad de permanecer en estado latente en el interior del hospedador, pudiendo replicarse y transmitirse durante los períodos de reactivación causados por procesos: de estrés, hormonales y de inmunodepresión (Carrasco & Almendral del Río, 2006; MacLachlan & Dubovi, 2011).

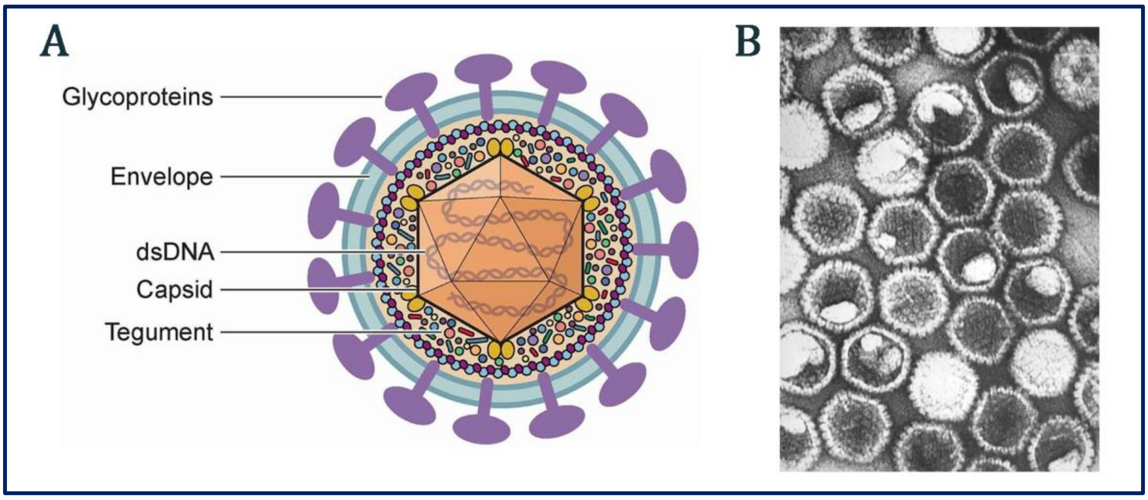


Figura 6: Panel de imágenes de viriones de herpesvirus realizado con el programa (Adobe Photoshop CS6, 2017). **A)** Estructura del virión de un *herpesvirus simplex.* La ilustración pertenece al siguiente artículo "*Pathogenesis and virulence of herpes simplex virus*" (Zhu & Viejo-Borbolla, 2021). **B)** Viriones de herpesvirus observados a través de microscopía electrónica. Imagen perteneciente a Centers for Disease Control and Prevention (CDC) (Fred Murphy & Sylvia Whitfield, 2020).

El orden de los Herpesvirales se encuentra compuesto por tres familias distintas: *Herpesviridae*, que afectan a los mamíferos, aves y reptiles; *Alloherpesviridae*, descrito en peces y anfibios; y *Malacoherpesviridae* identificado en los bivalvos, principalmente en ostras. A su vez la familia *Herpesviridae* se subdivide en tres subfamilias: *Alphaherpesvirinae*, *Betaherpesvirinae* y *Gammaherpesvirinae* (Davison y cols., 2009; Gatherer y cols., 2021; MacLachlan & Dubovi., 2011). Si bien hasta la fecha no se han descrito ningún caso de infección por betaherpesvirus (βHV) en cetáceos.

En los cetáceos se han hallado y descrito un amplio espectro de lesiones histopatológicas causadas por la infección de herpesvirus (HVs), pudiendo ir desde animales asintomáticos hasta la presentación de lesiones graves (Bellière y cols., 2010). Los *alphaherpesvirus* (αHV) se encuentran más relacionados con la aparición de infección sistémica grave, necrosis linfoide, nefritis intersticial, encefalitis y meningoencefalitis (Arbelo y cols., 2010, 2012; Blanchard y cols., 2001; Kennedy y cols., 1992; Sierra y cols., 2022; Sierra y cols., 2014; Van Elk y cols., 2016). Sin embargo, los *gammaherpesvirus* (УHV) producen lesiones cutáneas, mucocutáneas y genitales (Manire y cols., 2006; Saliki y cols., 2006; Seade y cols., 2017; Sierra y cols., 2015; Smolarek-Benson y cols., 2006; van Beurden y colsl., 2015) y además recientemente se han detectado en el Sistema nervioso central (SNC) (Sierra y cols., 2022; Vargas-Castro y cols., 2020).

En distintas especies de los miembros de la familia *Ziphiidae* se han identificado casos de infección tanto por αHV como por УHV; habiéndose descrito, en el caso concreto de la especie **zifio de Blainville,** un caso de infección por **αHV** en un animal varado en las Islas Canarias en el año 2004 asociado a una nefritis tubulointersticial necrotizante multifocal con cuerpos de inclusión intranucleares (Arbelo y cols., 2012), así como otro caso ocurrido en las costas atlánticas de Estados Unidos (EE.UU.) de infección por **yHV** también en el año 2004 asociada a una lesión de tipo focal papilomatosa en el pene (Saliki y cols., 2006). En la especie **zifio de Cuvier** se ha registrado un caso de infección por **αHV** en las Islas Canarias en el año 2005 asociada a una grave necrosis coagulativa y vasculitis fibrinonecrótica con cuerpos de inclusión intranucleares en células monocíticas del bazo y algunos linfonodos (Arbelo y cols., 2010), en Valencia en el año 2012 (Vargas-Castro y cols., 2021) y en las costas de Huelva en el año 2015 (Sierra y cols., 2022) sin que en estos dos últimos casos se detectaran lesiones claramente atribuibles a la infección por HV. En la especie **zifio de Stejneger** solamente se ha reportado un caso por infección por **yHV** en Japón entre los años 2001 y 2006 (Miyoshi y cols., 2011). Finalmente, también en la especie **zifio de Longman** solo se ha registrado un caso por infección por **αHV** en Hawái en el año 2010 (West y cols., 2013). En estos dos últimos casos tampoco se describen lesiones que se puedan atribuir específicamente a la infección por HV.

2.4.2. *MORBILLIVIRUS DE LOS DE CETÁCEOS* Y CASOS DETECTADOS EN ZIFIOS

El orden de los *Mononegavirales* se compone actualmente de un total de 11 familias: *Artoviridae, Bornaviridae, Filoviridae, Lispiviridae, Mymonaviridae, Nyamiviridae, Paramyxoviridae,* Pneumoviridae*, Rhabdoviridae, Sunviridae* y *Xinmoviridae* (Amarasinghe y cols., 2019). Sin embargo, este apartado solamente se centrará en la familia *Paramyxoviridae*, al ser uno de sus géneros objeto de estudio.

Los virus de la familia *Paramyxoviridae* se encuentran ampliamente distribuidos en la naturaleza y afectan a una gran variedad de especies de mamíferos, aves, reptiles y peces. Se caracterizan por producir una alta morbilidad y mortalidad al ser responsables de importantes enfermedades y epizootias, pudiendo representar un grave riesgo tanto para la salud animal como humana. Además, hay que resaltar que estos virus se caracterizan por tener la capacidad de producir infecciones cruzadas debido al contacto entre las especies de vida silvestre con animales domésticos y humanos, por lo que posiblemente pueden favorecer la aparición de nuevos paramixovirus no identificados (MacLachlan & Dubovi., 2017). Finalmente los miembros de esta familia se caracterizan por tener actividad linfotrópica, neurotrópica y epiteliotrópica (Carrasco & Almendral del Río., 2006b; MacLachlan & Dubovi., 2017).

Los viriones de la familia *Paramyxoviridae* presentan una estructura pleomórfica y se encuentran compuestos por una envoltura de naturaleza lipídica formada por glicoproteínas, en la que se encuentran la proteína matriz (M), la proteína de fusión (F) y la proteína de hemaglutinina (H) o hemaglutinina-neuraminidasa (NH). Además, presentan una nucleocápside en forma de espiga y helicoidalmente simétrica, en la que se encuentran el ARN viral y las siguientes proteínas: la nucleoproteína (N), la fosfoproteína (P) y la ARN polimerasa dependiente de ARN (L). Finalmente, el genoma se encuentra constituido por una molécula lineal de ARN monocatenario de polaridad negativa **(figura 7 A y B)** (MacLachlan & Dubovi., 2017; Rima y cols., 2019).

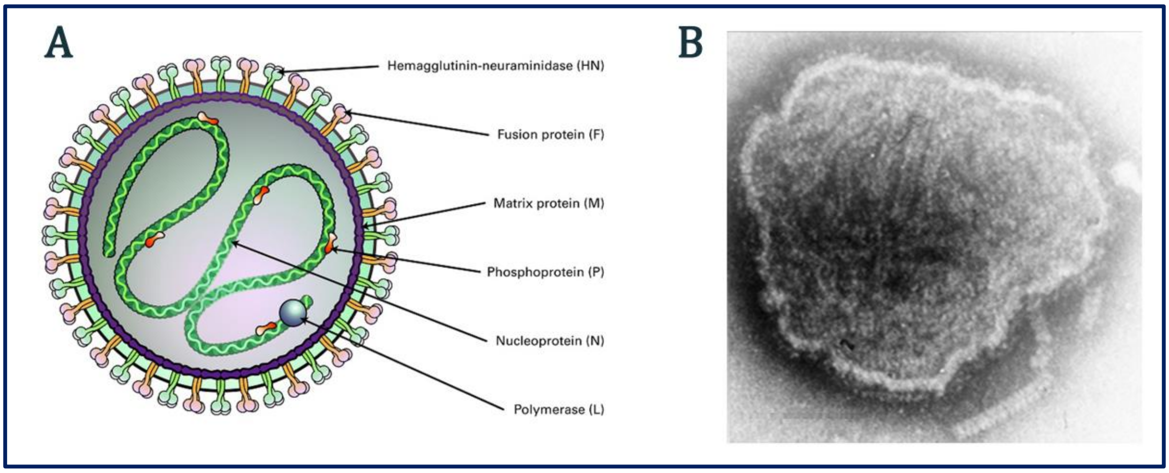


Figura 7: Panel de imágenes de la familia *Paramyxoviridae* realizado con el programa (Adobe Photoshop CS6, 2017). **A)** Estructura de un virión de la familia *Paramyxoviridae*, imagen obtenida de la web de LSBio, apartado “*Paramyxoviridae* (Mumps)” (LSBIO: Biotech Company, 2023). **B)** Microscopía electrónica de una partícula vírica del género *Morbillivirus*, imagen perteneciente al artículo “ICTV Virus Taxonomy Profile: *Paramyxoviridae*” (Rima y cols., 2019).

Taxonómicamente, la familia *Paramyxoviridae* se encuentra distribuida en las siguientes subfamilias: *Avulavirinae, Rubulavirinae, Orthoparamyxovirinae* y *Metaparamyxovirinae,* entre otras (Amarasinghe y cols., 2019; Rima y cols., 2019).

Esta tesis doctoral se centrará en la subfamilia *Orthoparamyxovirinae* y concretamente en su género *Morbillivirus* que contiene la especie el *morbillivirus de los cetáceos* (Amarasinghe y cols., 2019; Rima y cols., 2019).

El *morbillivirus de los cetáceos* (CeMV) (género *Morbillivirus*, subfamilia *Orthoparamyxovirinae*, familia *Paramyxoviridae*, orden *Mononegavirales*), se caracteriza por ser responsable de producir eventos de alta mortalidad a nivel mundial en los cetáceos, destacando las dos epizootias ocurridas en el Mar Mediterráneo entre los años 1990 al 1992 y 2006 al 2008 (Van Bressem y cols., 2014). Los hallazgos histopatológicos causados por el CeMV que se han descrito son: bronco-neumonía intersticial, depleción linfoide, encefalitis y meningoencefalitis no supurativa (De Vries y cols., 2015; Di Guardo y cols., 2005; Domingo y cols., 1992; Kennedy, 1998; Van Bressem y cols., 2014). Su contagio predispone a una mayor susceptibilidad de contraer co-infecciones, siendo los agentes más frecuentes descritos: HV (West y cols., 2013), *Brucella* spp. (West y cols., 2015), *Toxoplasma gondii* (Profeta y cols., 2015), *Aspergillus fumigatus* (Domingo y cols., 1992), entre otros. Además, este virus dentro del hospedador puede cursar como enfermedad sistémica aguda o sub-aguda, infección sistémica crónica, encefalitis localizada, o incluso como infección subclínica (Van Bressem y cols., 2014).

Por otro lado, también se han identificado dos tipos de linajes genéticos (Gulland y cols., 2018; Van Bressem y cols., 2014). Por un lado los **Linajes del hemisferio norte** (CeMV-1), en el que se incluirían las cepas del: *morbillivirus de los delfines* (DMV) (Visser y cols., 1993), *morbillivirus de las marsopas* (PMV) (Visser y cols., 1993), *morbillivirus de los calderones* (PWMV) (Taubenberger y cols., 2000) y el *morbillivirus de los zifios* (BWMV) (West y cols., 2013) y por otro lado, los **Linajes del hemisferio sur** (CeMV-2), que comprende otras cepas distintas a las anteriormente mencionadas y halladas en un Delfín costero (*Sotalia guianensis*) de Brasil (Groch y cols., 2014), en un delfín del Indo-pacifico en Australia (Stephens et al., 2014), y en un delfín de Fraser en Hawái (Jacob y cols., 2016).

En la actualidad, se han descrito los siguientes casos de infección por algunas de las cepas de CeMV en varias especies de la familia *Ziphiidae*. En la especie **zifio de Longman** se ha descrito un caso de infección por **BWMV** ocurrido en Hawái en el año 2010 (Jacob y cols., 2016; West y cols., 2013), así como otro caso de infección por **DMV** en Nueva Caledonia en el año 2013 (Garrigue y cols., 2016). En la especie **zifio de Cuvier** se han detectado casos de infección por **BWMV** en Hawái en el año 2008 (Jacob y cols., 2016); y por infección por **DMV** en Italia en el año 2015 (Centelleghe y cols., 2017). Finalmente, también se ha identificado un caso de infección por **BWMV** ocurrido en un **zifio de Blainville** nuevamente en Hawái en el año 2010 (Jacob y cols., 2016).

2.5. PATOLOGÍAS DE ORIGEN NATURAL PARASITARIO

2.5.1 *NASITREMA* SPP. Y CASOS DETECTADOS EN ZIFIOS

La clase Trematoda perteneciente al phylum de los Platyhelmintos y a la superclase Neodermata (WoRMS Editorial Board, 2022) se divide en dos subclases principales: los Monogeneas que presentan un ciclo de vida directo; y los Digeneas que para completar dicho ciclo requieren de la participación de un primer hospedador intermedio, principalmente un molusco. Los digeneos o duelas tienen especial relevancia en la medicina veterinaria, dado que las formas adultas suelen parasitar el intestino, los conductos biliares, los pulmones, los vasos sanguíneos y otros órganos de diferentes especies de vertebrados (cánidos, felinos, rumiantes, équidos y suidos) (Bowman, 2021; Gunn & Pitt, 2012; Taylor y cols., 2015).

Concretamente, dentro de la subclase Digenea se han identificado unas 54 especies agrupadas en cuatro familias (*Brauninide*, *Notocotylidae*, *Heterophyidae* y *Brachycladiidae*) capaces de parasitar a los cetáceos (Fraija-Fernández, 2016). Tiene especial relevancia la Familia *Brachycladiidae*, que se encuentra dividida en 10 géneros (*Brachycladium*, *Hunterotrema*, *Oschmarinella*, *Synthesium*, *Campula*, *Nasitrema* y *Cetitrema*, *Zalophotrema*, *Odhneriella* y *Orthosplanchnus*) (Fraija-Fernández y cols., 2016). El género *Nasitrema*, objeto de estudio en esta tesis, se compone de 10 especies: *N. delphini*, *N. globicephalae*, *N. attenuata*, *N. gondo*, *N. spathulatum*, *N. sunameri*, *N. lanceolata*, *N. lagenorhynchus*, *N. dalli*, y *N. stenosoma* (WoRMS Editorial Board, 2022).

Las especies de este género presentan un cuerpo con una morfología lanceolada o espatulada que, según la especie, puede variar desde formas redondeadas hasta más alargadas **(Figura 8)**. También pueden hallarse diferencias entre las mismas en cuanto a su extremo anterior, que puede exhibirse más marcadamente puntiagudo, o bien su extremo posterior, que se caracteriza por ser más largo y estrecho. Además, presentan una cutícula cubierta de espinas que recubre todo el cuerpo (Kikuchi y cols., 1987; Lim y colsl., 2016; Neiland y cols., 1970; Ozaki, 1935).

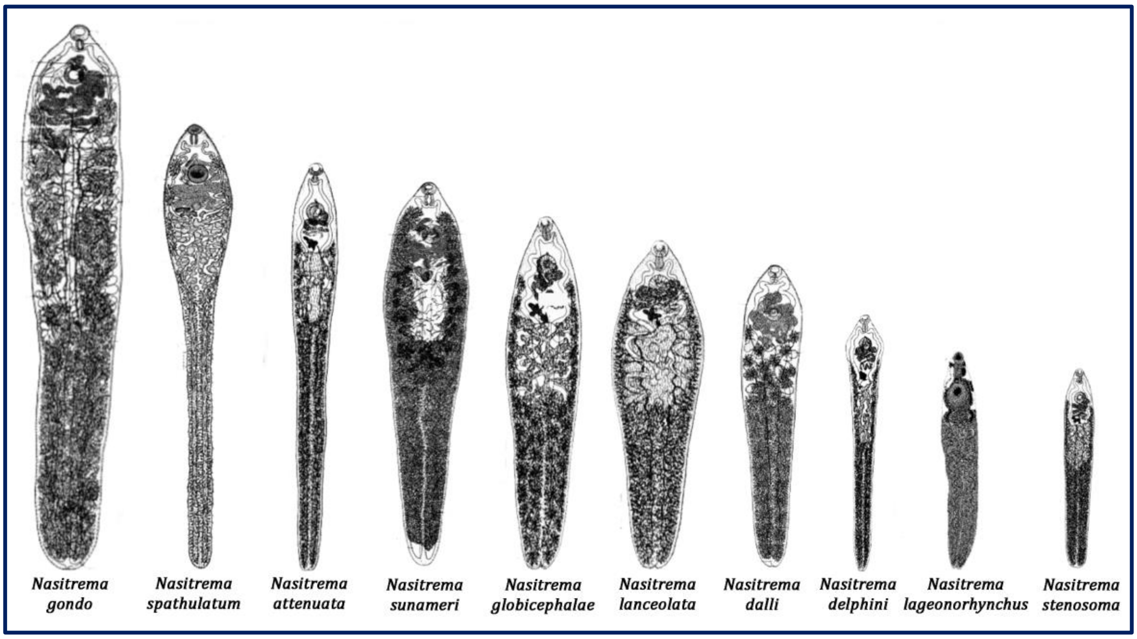


Figura 8: Panel de imágenes comparativo de las distintas especies que conforman el género *Nasitrema* realizado con el programa (Adobe Photoshop CS6, 2017). Imagen ilustrativa y modificada, obtenida del trabajo fin de máster titulado: "Prevalencia de tremátodos del género *Nasitrema* (Ozaki, 1935) en cetáceos varados en las Islas Canarias (2000 – 2018) e introducción a su detección inmunohistoquímica" de (García Oliva, 2019). A su vez dichas figuras pertenecen a los artículos de (Kikuchi y cols., 1987; Neiland y cols., 1970; Ozaki, 1935; Yamaguti, 1951).

En la porción anterior terminal del extremo anterior del trematodo **(Figura 9 A y B)**, se encuentra, a nivel ventral, la ventosa oral, que puede ser ovalada o redondeada. A continuación de esta ventosa presentan, por lo general, una pre-faringe corta seguida de una faringe, pudiendo haber diferencias y ser más marcadamente piriforme o alargada. De manera general al género *Nasitrema,* el esófago suele estar ausente. Posterior a la faringe se encuentra el intestino que se bifurca en dos ciegos intestinales, que se expanden por toda la longitud del cuerpo realizando circunvoluciones, desde la porción anterior hacia la posterior. El acetábulo se encuentra situado al final de la primera mitad del cuerpo y ventralmente en la línea media, que puede ser más ancho o largo, según la especie. Asimismo, en la cara anterior al acetábulo y en la línea media se halla el poro genital.

En cuanto a las estructuras reproductoras masculinas, correspondientes al cirro (presente o ausente), la pars prostática y la vesícula seminal se encuentran bien diferenciadas y se localizan entre el poro genital y el acetábulo. Asimismo, los testículos se hallan superpuestos lateralmente a los ciegos intestinales y se encuentran distribuidos en tándem, diferenciándose en testículo anterior y posterior, estando ramificados y lobulados y pudiendo ser más o menos marcadamente dendríticos. Con respecto a las estructuras reproductoras femeninas de *Nasitrema* spp., el útero presenta varias circunvoluciones intercecales o extracecales que avanzan por el margen izquierdo del acetábulo hasta el poro genital. El ovario está lobulado y situado ligeramente a la derecha de la línea media y localizado anterior a los testículos. En lo que se refiere a las glándulas vitelógenas o Vitellaria, éstas se encuentran divididas por numerosos acinos muy dendríticos que se extienden desde la cara anterior del testículo anterior o el acetábulo según la especie, hasta el extremo posterior ocupando toda su longitud. Por otro lado, en el extremo posterior se halla la vesícula excretora tubular que se extiende desde el orificio del poro excretor hasta la región del ovario y los testículos. En cuanto al poro excretor se trata de una apertura situada en la porción final del extremo posterior.

Finalmente, los huevos del género *Nasitrema* se caracterizan por su forma triangular u ovalada, por su color amarillo y por ser refringentes; además de presentar una cáscara gruesa con un extremo polar operculado.

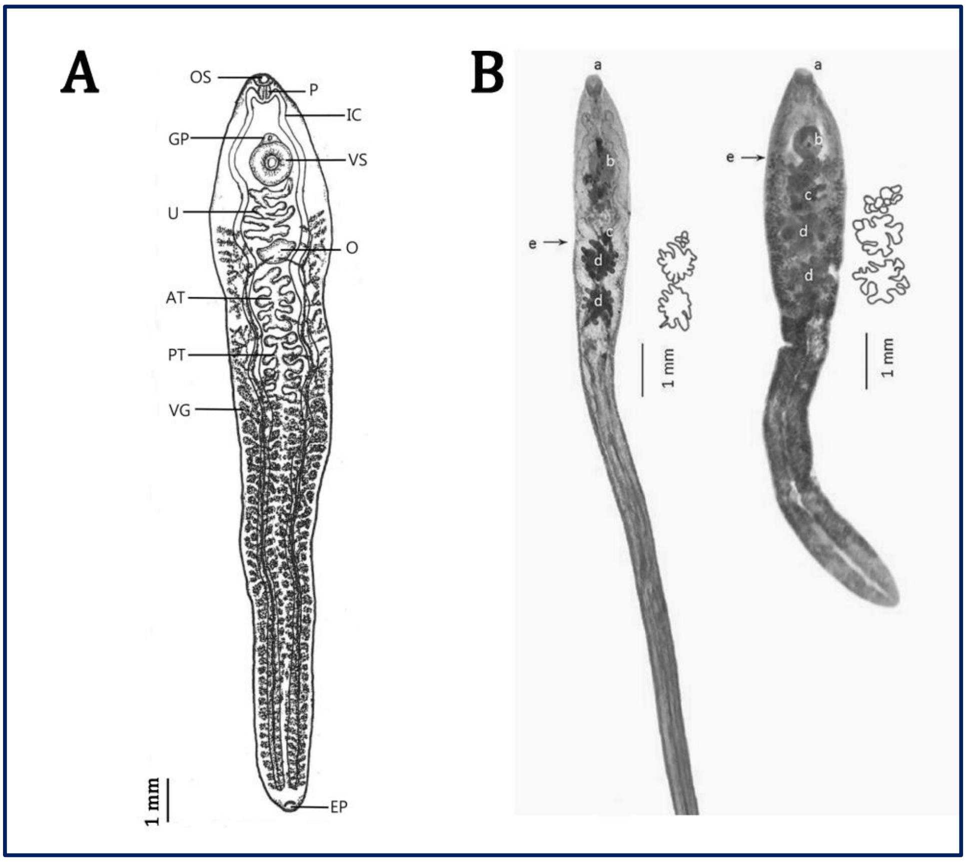


Figura 9: Morfología de *Nasitrema attenuata*. Panel de imágenes realizado con el programa (Adobe Photoshop CS6, 2017). **A)** Figura ilustrativa perteneciente al artículo de (Lim y cols., 2016) titulado: "*Nasitrema attenuata* (Digenia: Nasitrematidae) infection of long-beaked commondolphin (*Delphius capensis*) in the East Sea, Korea". Abreviaturas: (OS: ventosa oral; P: faringe; IC: ciego intestinal; GP: poro genital; VS: acetábulo; U: útero; O: ovario; AT: testículo anterior; PT: testículo posterior; VG: glándula vitelógenas o vitellaria; EP: poro excretor). **B)** Ejemplar obtenido en un *Sotalia guianensis,* imagen obtenida del artículo: "New records of *Nasitrema atenuatta* and *Nasitrema globicephalae* (Trematoda: Brachycladiidae) Neiland, Rice and Holden, 1970 in delphinids from South Atlantic" de (Ebert & Valente, 2013). Abreviaturas: (a: ventosa oral; b: acetábulo; c: ovario, d: testículos; e: glándulas vitelógenas o vitellaria.

Habitualmente en los cetáceos se han localizado ejemplares de *Nasitrema* spp. parasitando los senos pterigoideos, la cavidad timpánica, el oído medio e interno y el nervio vestibulococlear (VIII par craneal); además se ha descrito que el trematodo adulto puede realizar migraciones aberrantes llegando a alcanzar el cerebro (Arbelo y cols., 2013; Cowan y cols., 1986; Dailey, 1985; Dailey & Ridgway, 1976; Dailey & Walker, 1978; Degollada y cols., 2002; Díaz-Delgado y cols., 2018; Lewis & Berry, 1988; Lim y cols., 2016; Morimitsu y cols., 1987, 1992, 1986; Neiland y cols., 1970; O'Shea y cols., 1991; Oliveira y cols., 2011; Phillips & Suepaul, 2017; Ridgway & Murray, 1972; Schwab, 1985; Shiozaki & Amano, 2017; Sierra y cols., 2020; Walker & Cowan, 1981). Por lo que la presencia de trematodos adultos y sus huevos en estas regiones cefálicas cursa con diferentes tipos de lesiones: sinusitis, neuritis, otitis y meningoencefalitis (Arbelo y cols., 2013; Díaz-Delgado y cols., 2018; Morimitsu y cols., 1986, 1987, 1992; Ridgway & Murray, 1972; St. Leger y cols., 2018), las cuales imposibilitan la supervivencia de los animales afectados en la naturaleza, ya que dan lugar a la pérdida del equilibrio, incoordinación, disfunción de la ecolocalización, y son causa de eventos de varamientos activo (Dailey & Walker, 1978; Lewis & Berry, 1988; Morimitsu y cols., 1986, 1987, 1992; O'Shea y cols., 1991).

En la actualidad, el ciclo biológico de *Nasitrema* spp. sigue generando controversias, aunque recientemente se ha identificado que los ejemplares pertenecientes a la familia *Brachycladiidae* se encuentran más estrechamente relacionados con los miembros de la familia *Acanthocolpidae*. Los miembros de esta familia son trematodos propios de los peces teleósteos marinos que poseen ciclos de vida en los que intervienen un total de tres hospedadores, en donde los gasterópodos juegan un papel como primer hospedador intermediario; siendo los peces o los moluscos bivalvos los segundos hospedadores intermediarios (Kremnev y cols., 2020). Finalmente, la presencia de adultos y huevos de *Nasitrema attenuata* en las heces refuerza la hipótesis de que la infección podría adquirirse a través del consumo de pescado parasitado con alguno de los estadios larvarios del parásito (Kleinertz y cols., 2014; St. Leger y cols., 2018).

3. OBJETIVOS

Zifios de Sowerby (*Mesoplodon bidens*). FOTOGRAFÍA: © Karin Hartman/Nova Atlantis Foundation

3. OBJETIVOS

El propósito de este trabajo consistió en realizar un estudio retrospectivo para determinar las principales patologías infecciosas en los zifios varados en las costas de las Islas Canarias en un período de tiempo comprendido entre los años 1999 hasta el 2017. En base a las fuentes científicas descritas hasta la fecha actual, se toma como objetivo principal determinar, principalmente, la presencia de HVs (nuevos o ya conocidos), de CeMV, así como la presencia de posibles agentes patógenos no descritos anteriormente en los zifios, como es el caso de *Nasitrema* spp.

Para ello se tomaron como objetivos específicos:

1. Detectar, mediante técnicas moleculares, material genético de HV y CeMV en muestras de animales de la familia *Ziphiidae.*
2. Correlacionar, en los casos que sea posible, la positividad obtenida con la histopatología, identificando las lesiones compatibles con la infección causada por HV y CeMV.
3. Identificar, molecularmente, al patógeno asociado a una encefalitis piogranulomatosa y necrotizante multifocal crónica-activa grave en un zifio de Blainville.
4. Realizar análisis filogenéticos para analizar las relaciones entre las secuencias obtenidas con otras disponibles en éstas y otras especies.

4. RESUMEN DE LOS ARTÍCULOS PUBLICADOS A PARTIR DE ESTA TESIS

Zifio Calderón de Norte (*Hyperoodon ampullatus*). FOTOGRAFÍA: Laura Joan Feyrer y cols. 2019

4. RESUMEN DE LOS ARTÍCULOS PUBLICADOS A PARTIR DE ESTA TESIS

ARTÍCULO 1:

Título: Contribution to Herpesvirus Surveillance in Beaked Whales Stranded in the Canary Islands.

Autores: Idaira Felipe-Jiménez, Antonio Fernández, Marisa Andrada, Manuel Arbelo, Simone Segura-Göthlin, Ana Colom-Rivero y Eva Sierra.

Revista: *Animals* 11, no. 7: 1923

Año: 2021

DOI: https://doi.org/10.3390/ani11071923

Resumen: Los herpesvirus (HVs) (Subfamilia *Alpha* y *Gammaherpesvirinae*) se han detectado en diversas especies de cetáceos con diferentes implicaciones patológicas. Sin embargo, la información de la que se dispone sobre su presencia en los zifios es todavía escasa. En este estudio se analizaron 55 zifios (35 *Ziphius cavirostris* y 20 ejemplares del género *Mesoplodon*) varados en las costas de las Islas Canarias desde el año 1999 al año 2017. En total se examinaron 294 muestras de tejidos frescos preservados en congelación (-80ºC) en las instalaciones del Instituto Universitario de Sanidad Animal y Seguridad Alimentaria de la Universidad de Las Palmas de Gran Canaria, que incluían, según la disponibilidad en cada caso: pulmón (16,33%; (48/294)), riñón (15,65%; (46/294)), cerebro (12,93%; (38/294)), piel (12,59%, (37/294)), hígado (12,24%; (36/294)), bazo (9,86%; (29/294)), linfonodo mesentérico (8,16% (24/294)), músculo esquelético (6,12%; (18/294)), intestino (2,04%; (6/294)), linfonodo preescapular (1,36%; (4/294)), linfonodo mediastínico (0,68%; (2/294)), glándula tiroidea (0,34%; (1/294)), timo (0,34%; (1/294)), paladar blando (0,34%; (1/294)), esófago (0,34%; (1/294)) y sangre (0,34%; (1/294)). A partir de dichas muestras disponibles se realizó simultáneamente la extracción de ADN/ARN. La detección molecular de HV se realizó mediante una PCR convencional anidada basada en el gen de la ADN polimerasa. Tanto en el protocolo de extracción como en el de PCR se incluyeron un control negativo y un control positivo de amplificación (ADN de un herpesvirus conocido obtenido previamente en nuestro laboratorio). Para la visualización de los amplicones se realizó una electroforesis horizontal en gel de agarosa al 2% con tinción GelRed®. Además, a los animales que resultaron ser positivos para HV se les realizó una PCR a tiempo real con transcripción inversa (RT-qPCR) basada en el fluoróforo SYBR® Green para la detección del *morbilivirus de los cetáceos* (CeMV). Como resultado, se detectó material genético de HV en 14,45% (8/55) de los zifios analizados, incluyendo 2 animales previamente confirmados como positivos. Un porcentaje de positividad del

8,57% fue hallado en el grupo de los zifios de Cuvier, mientras que el porcentaje de positividad se incrementó hasta un 25% dentro del grupo del género *Mesoplodon* (tres zifios de Blainville (*Mesoplodon densirostris*), un zifio de Gervais (*Mesoplodon europaeus*) y un zifio de Sowerby (*Mesoplodon bidens*)). Todas las secuencias obtenidas en este estudio pertenecieron a la subfamilia *Alphherpesvirinae,* de las cuáles tres fueron consideradas secuencias noveles e identificadas dentro del grupo del género *Mesoplodon*, siendo además negativas a CeMV. El estudio filogenético mostró que, analizadas por especies, las secuencias detectadas en la especie zifio de Cuvier resultaron estar más próximas a secuencias detectadas en delfines listados (*Stenella coeruleoalba*) varados en el Mar Mediterráneo y las costas del Atlántico central y nororiental (Canarias y Portugal) y a una secuencia detectada en otro zifio de Cuvier varado en el Mar Mediterráneo. Esta relación podría indicar que la transmisión de HV se produce entre distintas especies de regiones próximas, tal y como algunos autores ya había sugerido anteriormente. Las secuencias detectadas en la especie zifio de Blainville se encuentran, por lo general, más relacionadas con una secuencia detectada en el cerebro de un delfín listado varado en el Mar Mediterráneo en 2011, si bien una de las secuencias se considera novel, relacionándose con una secuencia obtenida de una lesión cutánea del pene de una ballena beluga (*Delphinapterus leucas*) varada en el estuario de San Lorenzo (Canadá), la cual, a su vez, también se había considerado novel y nombrada tentativamente como herpesvirus de la ballena beluga. Dos secuencias noveles se obtuvieron de animales de las especies zifio de Gervais y de Sowerby, respectivamente, si bien mostraron la relación más cercana con secuencias detectadas en otras especies de la familia *Ziphiidae*, por lo que se sugiere que interacciones interespecíficas entre estas cuatro especies de zifios analizadas en nuestro estudio podría explicar la transmisión de HV dentro del familia *Ziphiidae*. Además, según nuestro conocimiento, esta es la primera descripción de casos de infección por HVs en un zifio de Gervais y un zifio de Sowerby. La aparición de nuevas secuencias de HVs en miembros de la familia Ziphiidae aportará un mayor conocimiento sobre la coevolución de los HVs en estas especies de cetáceos. Tres de ocho zifios positivos a HV mostraron lesiones histopatológicas indicativas de replicación viral activa, destacando la presencia de nefritis tubulointersticial necrotizante con cuerpos de inclusión intranucleares en dos zifios de Blainville que vararon con 15 años de diferencias. **(Figura 10).**

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Contribution to Herpesvirus Surveillance in Beaked Whales Stranded in the Canary Islands

Idaira Felipe-Jiménez, Antonio Fernández, Marisa Andrada *, Manuel Arbelo, Simone Segura-Göthlin, Ana Colom-Rivero and Eva Sierra

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Simple Summary: Herpesviruses (HVs) are a large family of DNA viruses infecting animals (including insects and mollusks) and humans. Cetaceans can be also infected by HVs presenting different range of lesions, from dermatitis to meningoencephalitis, or being asymptomatic. Several studies have addressed the question of HVs in cetaceans, although no previous systematic survey of HV in beaked whales (BWs) (*Ziphiidae* family) has been previously performed. The family *Ziphiidae*, which includes 22 species in 6 genera, is one of the most widespread families of cetaceans, with a strict oceanic habitat pattern. Beaked whales, Cuvier's BW in particular, are one of the deepest diving whales and are of particular interest because of a notable relationship between military operations employing mid-frequency sonar and the mass stranding of BWs in different geographic areas, including the Canary Islands. In this study, we analyzed 55 BWs (294 samples) stranded in the Canary Islands from 1990 to 2017 by molecular methods (conventional nested polymerase chain reaction). Our results showed that 8 BWs were infected by HVs, although only three animals displayed lesions indicative of active viral replication. Phylogenetic analysis suggests that HV-BW sequences are species-specific, although more studies are needed to better address this question.

Abstract: Herpesviruses (HVs) (*Alpha-* and *Gammaherpesvirinae* subfamilies) have been detected in several species of cetaceans with different pathological implications. However, available information on their presence in beaked whales (BWs) is still scarce. In this study, a total of 55 BWs (35 *Ziphius cavirostris* and 20 animals belonging to the *Mesoplodon* genus) were analyzed. Samples (n = 294) were obtained from BWs stranded along the coasts of the Canary Islands (1990–2017). Molecular detection of HV was performed by means of a conventional nested PCR based on the DNA polymerase gene. Herpesvirus was detected in 14.45% (8/55) of the analyzed BWs, including 2 positive animals from a previous survey. A percentage positivity of 8.57% was found within the Cuvier's BW group, while the percentage of positivity rose to 25% within the *Mesoplodon* genus group (three *M. densirostris,* one *M. europaeus*, and one *M. bidens*). All the obtained sequences from this study belonged to the *Alphaherpesvirinae* subfamily, from which three are considered novel sequences, all of them within the *Mesoplodon* genus group. In addition, to our knowledge, this is the first description of HV infection in Gervais' and Sowerby's BWs. Three out of eight HV-positive BWs displayed histopathological lesions indicative of active viral replication.

Keywords: herpesvirus; alphaherpesvirus; DNA polymerase; PCR; beaked whale; *Ziphius cavirostris*; *Mesoplodon*; cetaceans

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Citation: Felipe-Jiménez, I.; Fernández, A.; Andrada, M.; Arbelo, M.; Segura-Göthlin, S.; Colom-Rivero, A.; Sierra, E. Contribution to Herpesvirus Surveillance in Beaked Whales Stranded in the Canary Islands. *Animals* **2021**, *11*, 1923. https://doi.org/10.3390/ani11071923

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1. Introduction

The family *Ziphiidae* is one of the most widespread families of cetaceans, with a strict oceanic habitat pattern. The Canary Islands, due to its volcanic origin and oceanic location, hold resident and transient populations of Beaked whales (BWs), with records of six

Animals **2021**, *11*, 1923. https://doi.org/10.3390/ani11071923 https://www.mdpi.com/journal/animals

Figura 10: Portada del primer artículo de esta tesis doctoral titulado "Contribution to Herpesvirus Surveillance in Beaked Whales Stranded in the Canary Islands".

ARTÍCULO 2:

Título: Molecular Diagnosis of *Cetacean Morbillivirus* in Beaked Whales Stranded in the Canary Islands (1999–2017).

Autores: Idaira Felipe-Jiménez, Antonio Fernández, Manuel Arbelo, Simone Segura-Göthlin, Ana Colom-Rivero, Cristian M. Suárez-Santana, Jesús De La Fuente, and Eva Sierra.

Revista: *Veterinary Sciences* 9, no. 3: 121

Año: 2022

DOI: https://doi.org/10.3390/vetsci9030121

Resumen: El *morbillivirus de los cetáceos* (CeMV) se trata de un virus compuesto por una sola cadena de ARN, que es responsable de causar eventos de epizootia a nivel mundial con altas tasas de mortalidad en los cetáceos, si bien la información de la que se dispone con respecto a los zifios es aún escasa, con solo unos pocos casos descritos a nivel mundial. Concretamente se han descrito varios casos de infección por la cepa del *morbillivirus de los zifios* (BWMV) en las especies zifio de Longman (*Indopacetus pacificus*), zifio de Blainville (*Mesoplodon densirostris*) y zifio de Cuvier (*Ziphius cavirostris*) en Hawái y por la cepa *morbillivirus del delfín* (DMV) en la especie zifio de Longman en Nueva Caledonia y en el zifio de Cuvier en Italia. En este trabajo se realizó un estudio retrospectivo para la detección de CeMV en muestras tisulares de zifios varados en las Islas Canarias entre los años 1999 hasta 2017. Específicamente se analizaron un total de 319 muestras de tejidos frescos preservados en congelación (-80ºC) en las instalaciones del Instituto Universitario de Sanidad Animal y Seguridad Alimentaria de la Universidad de Las Palmas de Gran Canaria, pertenecientes a 55 zifios (35 zifios de Cuvier y 20 ejemplares pertenecientes al género *Mesoplodon*). Las muestras, según la disponibilidad en cada caso, incluían: piel (50/319; 15,67%), pulmón (49/319; 15,36%), hígado (47/319; 14,73%), riñón (46/319; 14,42 %), cerebro (38/319; 11,91 %), bazo (29/319; 9,09 %), ganglio linfático mesentérico (24/319; 7,52 %), músculo esquelético (18/319; 5,64 %;), intestino (6/319; 1,88 %;), ganglio linfático preescapular (4/319; 1,25%), ganglio linfático mediastínico (2/319; 0,63%), glándula tiroides (1/319; 0,31%), timo (1/319; 0,31%), paladar (1/319; 0,31%), esófago (1/319; 0,31%), pene (1/319; 0,31%) y sangre (1/319; 0.31%). La detección molecular de CeMV se realizó mediante la amplificación de fragmentos de los genes de la proteína de fusión (F) y la fosfoproteína (P) del CeMV a través, principalmente, de una o varias de las siguientes reacciones en cadena de la polimerasa (PCR): (1) una PCR convencional modificada con un paso de transcripción inversa (RT-PCR) que amplificó un fragmento de la región conservada del gen de la P, (2) una PCR a tiempo real con transcripción inversa (RT-qPCR) que amplificó una región del gen de la F y (3) una PCR a tiempo real con un paso de transcripción inversa (PAN RT-qPCR) basada en el fluoróforo SYBR® Green y que amplifica una región del gen de la P, presentado una alta sensibilidad para detectar con éxito las cepas del DMV, *morbillivirus de los calderones* (PWMV) y el *morbillivirus del delfín costero* (GDMV). Además, se añadieron un control negativo y un control positivo de amplificación (ARN de CeMV conocido obtenido previamente en nuestro laboratorio). La integridad del ARN no se pudo demostrar en algunas muestras, concretamente en 11 animales. Adicionalmente, en todas las muestras se comprobó la presencia de herpesvirus mediante una PCR de panherpesvirus anidada convencional basada en el gen del ADN polimerasa. La positividad a la cepa DMV se detectó, exclusivamente, en una muestra de piel de un macho subadulto de zifio de Cuvier varado en el año 2002. Este hallazgo supone la detección más temprana confirmada de DMV en esta especie, 13 años antes con respecto a la primera descripción de DMV detectada a partir de una muestra de pulmón en una cría de zifio de Cuvier varada en Italia en 2015. La obtención de la secuencia del gen P mostró un porcentaje de 100% de identidad con respecto a otra secuencia de DMV detectada en un delfín listado (*Stenella coeruleoalba*) varado en las Islas Canarias en el mismo año, indicando la existencia de una infección cruzada entre especies como previamente ya han sugerido otros autores. Además, el análisis filogenético apoyó la hipótesis previa de la presencia de una circulación endémica de una cepa de DMV en el Océano Atlántico similar a las detectada posteriormente en el Atlántico nororiental, en el mar Mediterráneo y en el Pacífico sudoccidental. Por tanto, estos resultados obtenidos también indicarían que el CeMV se encuentra de manera endémica en el Océano Atlántico central y nororiental, y que la transmisión del virus entre animales podría ocurrir a través del Estrecho de Gibraltar. Este intercambio viral podría causar brotes en el Mediterráneo con eventos de mortalidad inusuales en esa región debido a la alta densidad de animales con comportamiento gregario, especialmente en delfines listados. En cuanto a otras especies de cetáceos, los zifios de Cuvier presentan una distribución mundial y se encuentran en casi todas las aguas templadas, subtropicales, tropicales, subpolares y polares. Además, presentan patrones de comportamientos migratorios en el Océano Atlántico y probablemente podrían actuar como reservorios de propagación y dispersión del virus hacia otras poblaciones cetáceos más susceptibles **(Figura 11)**.

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Article

Molecular Diagnosis of Cetacean Morbillivirus in Beaked Whales Stranded in the Canary Islands (1999–2017)

Idaira Felipe-Jiménez, Antonio Fernández *, Manuel Arbelo, Simone Segura-Göthlin, Ana Colom-Rivero, Cristian M. Suárez-Santana, Jesús De La Fuente and Eva Sierra

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Abstract: A retrospective survey for detecting the cetacean morbillivirus (CeMV) was carried out in beaked whales (BWs) stranded in the Canary Islands (1999–2017). CeMV is responsible for causing worldwide epizootic events with the highest mass die-offs in cetaceans, although the epidemic status of the Canarian Archipelago seems to be that of an endemic situation. A total of 319 tissue samples from 55 BWs (35 Cuvier's BWs and 20 specimens belonging to the *Mesoplodon* genus) were subjected to the amplification of a fragment of the fusion protein (F) and/or phosphoprotein (P) genes of CeMV by means of one or more of three polymerase chain reactions (PCR). RNA integrity could not be demonstrated in samples from 11 animals. Positivity (dolphin morbillivirus strain (DMV)) was detected in the skin sample of only a subadult male Cuvier's BW stranded in 2002, being the earliest confirmed occurrence of DMV in the Cuvier's BW species. The obtained P gene sequence showed the closest relationship with other DMVs detected in a striped dolphin stranded in the Canary Islands in the same year. A phylogenetic analysis supports a previous hypothesis of a cross-species infection and the existence of the circulation of endemic DMV strains in the Atlantic Ocean similar to those later detected in the North-East Atlantic, the Mediterranean Sea and the South-West Pacific.

Keywords: cetaceans; morbillivirus; beaked whales; Canary Islands; PCR; *Ziphius cavirostris*; *Ziphiidae*

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Citation: Felipe-Jiménez, I.; Fernández, A.; Arbelo, M.; Segura-Göthlin, S.; Colom-Rivero, A.; Suárez-Santana, C.M.; De La Fuente, J.; Sierra, E. Molecular Diagnosis of Cetacean Morbillivirus in Beaked Whales Stranded in the Canary Islands (1999–2017). *Vet. Sci.* **2022**, *9*, 121. https://doi.org/10.3390/vetsci9030121

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1. Introduction

The cetacean morbillivirus (CeMV; genus *Morbillivirus*, *Paramyxoviridae* family, order *Mononegavirales*), consisting of a single linear molecule of negative-sense single-stranded RNA, has been responsible for major epizootic diseases in cetaceans, causing many of the biggest mass die-offs worldwide in these species [1]. The main pathological findings described in infected cetaceans are broncho-interstitial pneumonia, lymphoid depletion and nonsuppurative meningoencephalitis, as well as an increased susceptibility to opportunistic infections [1–5]. According to the stage of the infection, four presentation forms of the disease have been recently described [1]: acute and subacute systemic diseases, chronic systemic infections and chronic localized CeMV encephalitis.

Two lineages of CeMV have been proposed [1,6]: CeMV-1 for the "old" northern hemisphere lineage that includes dolphin morbillivirus (DMV) [7], porpoise morbillivirus (PMV) [7], pilot-whale morbillivirus (PWMV) [8] and beaked-whale morbillivirus (BWMV) [9] strains; and CeMV-2 for the "new" southern hemisphere lineage, consisting of virus strains detected in a Guiana dolphin (*Sotalia guianensis*) from Brazil [10] and in Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) from Western Australia [11]. In addition, it has been recently reported that a novel morbillivirus was detected in a Fraser's dolphin (*Lagenodelphis hosei*) stranded in Hawaii that is dissimilar to the BWMV previously identi-

Figura 11: Portada del segundo artículo de esta tesis doctoral titulado "Molecular Diagnosis of *Cetacean Morbillivirus* in Beaked Whales Stranded in the Canary Islands (1999–2017)"

ARTÍCULO 3:

Título: Cerebral nasitremiasis in a Blainville's beaked whale (*Mesoplodon densirostris*) stranded in the Canary Islands.

Autores: Idaira Felipe-Jiménez, Antonio Fernández, Yara Bernaldo de Quirós, Marina Arregui-Gil, Raquel Puig-Lozano, Manuel Arbelo, Eva Sierra.

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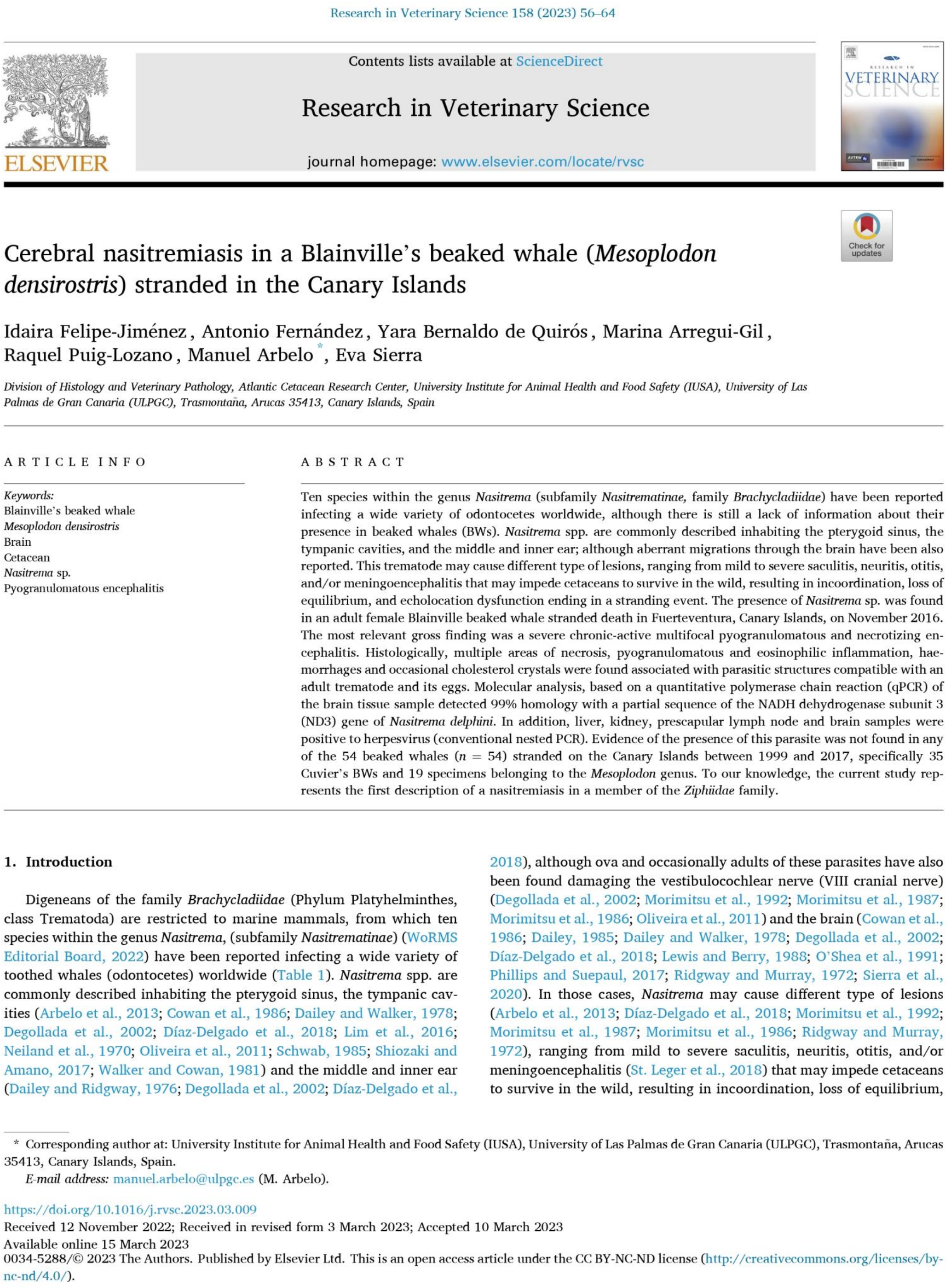
Resumen:

El género *Nasitrema* (subfamilia *Nasitrematinae*, familia *Brachycladiidae*) se encuentra constituido por diez especies diferentes capaces de parasitar a una amplia variedad de odontocetos a nivel mundial, si bien no hay descripciones previas sobre su presencia en zifios. Infestaciones por *Nasitrema* spp. se ha descrito normalmente en los senos pterigoideos, la cavidad timpánica y el oído medio e interno; aunque también se ha reportado la existencia de migraciones aberrantes hasta alcanzar el cerebro. Por lo que la presencia de trematodos adultos y sus huevos en estas regiones cefálicas puede causar una amplia gama de lesiones, que van desde sinusitis leve a severa, neuritis, otitis y/o meningoencefalitis lo que impide que los cetáceos puedan sobrevivir en la naturaleza, ya que produce falta de coordinación, pérdida del equilibrio y disfunción en la ecolocalización, lo que conlleva a eventos de varamiento. En este trabajo describimos el hallazgo de una nasitremiasis cerebral en una hembra adulta de zifio de Blainville varada en Fuerteventura, Islas Canarias, en noviembre del año 2016. El hallazgo macroscópico más relevante fue una encefalitis piogranulomatosa y necrotizante multifocal crónica-activa grave. El estudio histopatológico del sistema nervioso central evidenció múltiples áreas de necrosis, inflamación piogranulomatosa y eosinofílica, hemorragias y ocasionales cristales de colesterol asociados a estructuras parasitarias compatibles con un trematodo adulto y sus huevos. El análisis molecular, basado en la reacción en cadena de la polimerasa cuantitativa (qPCR) de una muestra de tejido cerebral, detectó un 99 % de homología con una secuencia parcial del gen de la subunidad 3 de la NADH deshidrogenasa (ND3) de la especie *N. delphini*. Además, las muestras de hígado, riñón, ganglio preescapular y cerebro resultaron positivas a herpesvirus (PCR anidada convencional). Asimismo, no se encontraron evidencias de la presencia de este parásito en ningún otro zifio (n = 54) varado en Canarias entre los años 1999 y 2017; en concreto en 35 zifios de Cuvier y 19 ejemplares pertenecientes al género *Mesoplodon*. Hasta donde sabemos, el presente estudio representa la primera descripción de una nasitremiasis en un miembro de la familia *Ziphiidae* **(Figura 12)**.



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Cerebral nasitremiasis in a Blainville's beaked whale (*Mesoplodon densirostris*) stranded in the Canary Islands

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ABSTRACT

Ten species within the genus *Nasitrema* (subfamily *Nasitrematinae,* family *Brachycladiidae*) have been reported infecting a wide variety of odontocetes worldwide, although there is still a lack of information about their presence in beaked whales (BWs). *Nasitrema* spp. are commonly described inhabiting the pterygoid sinus, the tympanic cavities, and the middle and inner ear; although aberrant migrations through the brain have been also reported. This trematode may cause different type of lesions, ranging from mild to severe saculitis, neuritis, otitis, and/or meningoencephalitis that may impede cetaceans to survive in the wild, resulting in incoordination, loss of equilibrium, and echolocation dysfunction ending in a stranding event. The presence of *Nasitrema* sp. was found in an adult female Blainville beaked whale stranded death in Fuerteventura, Canary Islands, on November 2016. The most relevant gross finding was a severe chronic-active multifocal pyogranulomatous and necrotizing encephalitis. Histologically, multiple areas of necrosis, pyogranulomatous and eosinophilic inflammation, haemorrhages and occasional cholesterol crystals were found associated with parasitic structures compatible with an adult trematode and its eggs. Molecular analysis, based on a quantitative polymerase chain reaction (qPCR) of the brain tissue sample detected 99% homology with a partial sequence of the NADH dehydrogenase subunit 3 (ND3) gene of *Nasitrema delphini*. In addition, liver, kidney, prescapular lymph node and brain samples were positive to herpesvirus (conventional nested PCR). Evidence of the presence of this parasite was not found in any of the 54 beaked whales ($n = 54$) stranded on the Canary Islands between 1999 and 2017, specifically 35 Cuvier's BWs and 19 specimens belonging to the *Mesoplodon* genus. To our knowledge, the current study represents the first description of a nasitremiasis in a member of the *Ziphiidae* family.

1. Introduction

Digeneans of the family *Brachycladiidae* (Phylum Platyhelminthes, class Trematoda) are restricted to marine mammals, from which ten species within the genus *Nasitrema*, (subfamily *Nasitrematinae*) (WoRMS Editorial Board, 2022) have been reported infecting a wide variety of toothed whales (odontocetes) worldwide (Table 1). *Nasitrema* spp. are commonly described inhabiting the pterygoid sinus, the tympanic cavities (Arbelo et al., 2013; Cowan et al., 1986; Dailey and Walker, 1978; Degollada et al., 2002; Díaz-Delgado et al., 2018; Lim et al., 2016; Neiland et al., 1970; Oliveira et al., 2011; Schwab, 1985; Shiozaki and Amano, 2017; Walker and Cowan, 1981) and the middle and inner ear (Dailey and Ridgway, 1976; Degollada et al., 2002; Díaz-Delgado et al., 2018), although ova and occasionally adults of these parasites have also been found damaging the vestibulocochlear nerve (VIII cranial nerve) (Degollada et al., 2002; Morimitsu et al., 1992; Morimitsu et al., 1987; Morimitsu et al., 1986; Oliveira et al., 2011) and the brain (Cowan et al., 1986; Dailey, 1985; Dailey and Walker, 1978; Degollada et al., 2002; Díaz-Delgado et al., 2018; Lewis and Berry, 1988; O'Shea et al., 1991; Phillips and Suepaul, 2017; Ridgway and Murray, 1972; Sierra et al., 2020). In those cases, *Nasitrema* may cause different type of lesions (Arbelo et al., 2013; Díaz-Delgado et al., 2018; Morimitsu et al., 1992; Morimitsu et al., 1987; Morimitsu et al., 1986; Ridgway and Murray, 1972), ranging from mild to severe saculitis, neuritis, otitis, and/or meningoencephalitis (St. Leger et al., 2018) that may impede cetaceans to survive in the wild, resulting in incoordination, loss of equilibrium,

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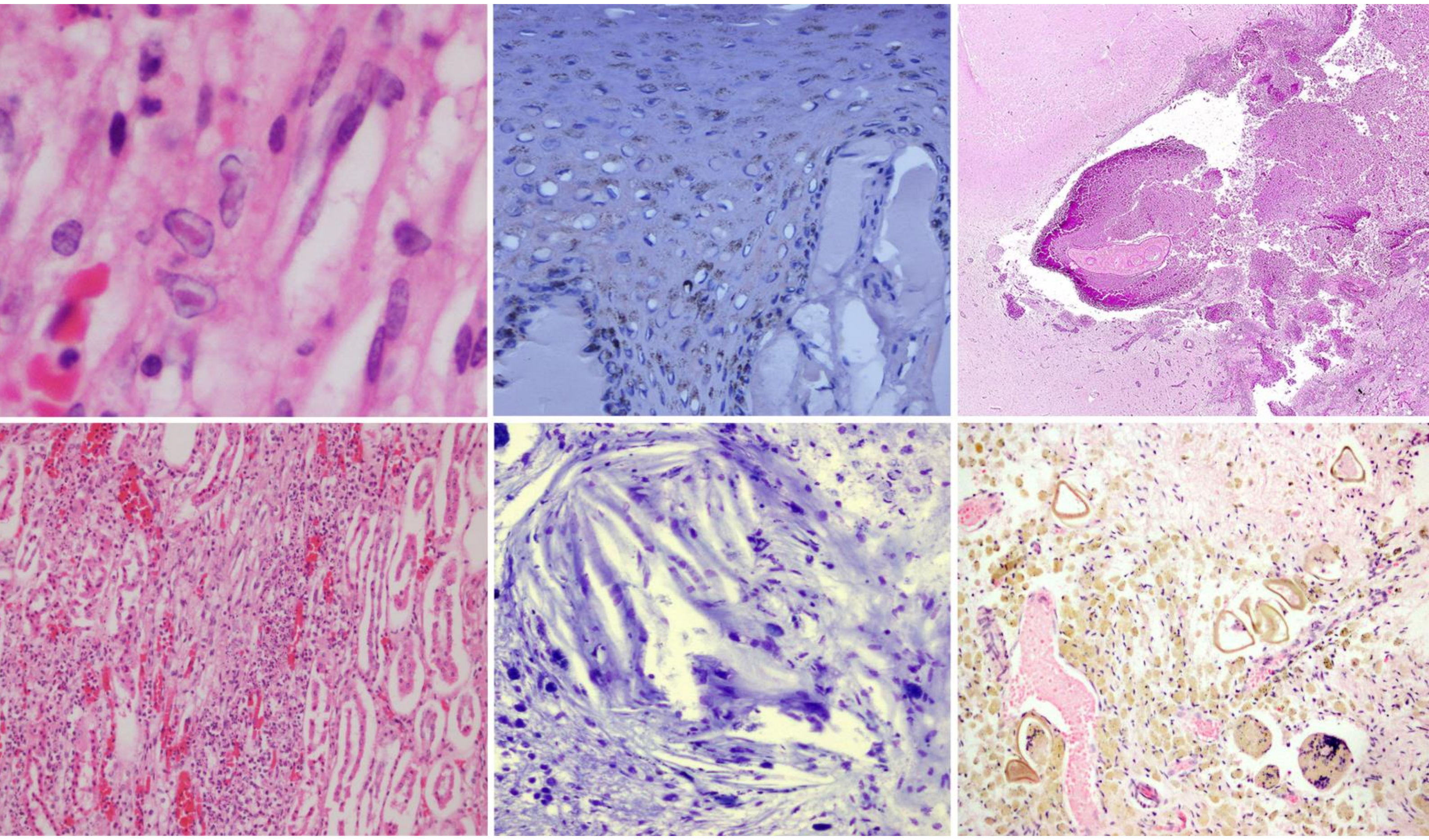
Figura 12: Portada del tercer artículo de esta tesis doctoral titulado "Cerebral nasitremiasis in a Blainville's beaked whale (*Mesoplodon densirostris*) stranded in the Canary Islands".

5. RESULTADOS



5.1. ARTÍCULO 1:

Contribution to Herpesvirus Surveillance in Beaked Whales Stranded in the Canary Islands.

animals

MDPI

Article

Contribution to Herpesvirus Surveillance in Beaked Whales Stranded in the Canary Islands

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Simple Summary: Herpesviruses (HVs) are a large family of DNA viruses infecting animals (including insects and mollusks) and humans. Cetaceans can be also infected by HVs presenting different range of lesions, from dermatitis to meningoencephalitis, or being asymptomatic. Several studies have addressed the question of HVs in cetaceans, although no previous systematic survey of HV in beaked whales (BWs) (*Ziphiidae* family) has been previously performed. The family *Ziphiidae*, which includes 22 species in 6 genera, is one of the most widespread families of cetaceans, with a strict oceanic habitat pattern. Beaked whales, Cuvier's BW in particular, are one of the deepest diving whales and are of particular interest because of a notable relationship between military operations employing mid-frequency sonar and the mass stranding of BWs in different geographic areas, including the Canary Islands. In this study, we analyzed 55 BWs (294 samples) stranded in the Canary Islands from 1990 to 2017 by molecular methods (conventional nested polymerase chain reaction). Our results showed that 8 BWs were infected by HVs, although only three animals displayed lesions indicative of active viral replication. Phylogenetic analysis suggests that HV-BW sequences are species-specific, although more studies are needed to better address this question.

Abstract: Herpesviruses (HVs) (*Alpha*- and *Gammaherpesvirinae* subfamilies) have been detected in several species of cetaceans with different pathological implications. However, available information on their presence in beaked whales (BWs) is still scarce. In this study, a total of 55 BWs (35 *Ziphius cavirostris* and 20 animals belonging to the *Mesoplodon* genus) were analyzed. Samples (n = 294) were obtained from BWs stranded along the coasts of the Canary Islands (1990–2017). Molecular detection of HV was performed by means of a conventional nested PCR based on the DNA polymerase gene. Herpesvirus was detected in 14.45% (8/55) of the analyzed BWs, including 2 positive animals from a previous survey. A percentage positivity of 8.57% was found within the Cuvier's BW group, while the percentage of positivity rose to 25% within the *Mesoplodon* genus group (three *M. densirostris*, one *M. europaeus*, and one *M. bidens*). All the obtained sequences from this study belonged to the *Alphaherpesvirinae* subfamily, from which three are considered novel sequences, all of them within the *Mesoplodon* genus group. In addition, to our knowledge, this is the first description of HV infection in Gervais' and Sowerby's BWs. Three out of eight HV-positive BWs displayed histopathological lesions indicative of active viral replication.

Keywords: herpesvirus; alphaherpesvirus; DNA polymerase; PCR; beaked whale; *Ziphius cavirostris*; *Mesoplodon*; cetaceans

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1. Introduction

The family *Ziphiidae* is one of the most widespread families of cetaceans, with a strict oceanic habitat pattern. The Canary Islands, due to its volcanic origin and oceanic location, hold resident and transient populations of Beaked whales (BWs), with records of six different species. Beaked whales, Cuvier's BW in particular, are one of the deepest diving whales. Depths of more than 2900 m and dive durations of over 2 h have been recently recorded in Cuviers' BWs during single breath-hold dives [1]. Most of the distribution information of BWs is based on stranding records. They face threats from entanglement in fishing gear, ingestion of marine debris, and ship collision, among others [2–4]. They are also of particular interest because of a notable relationship between military operations employing mid-frequency sonar and the mass stranding of BWs in different geographic areas, including the Canary Islands [5–8]. Natural pathologies affecting these species include verminous arteritis by *Crassicauda* spp. [9], brucellosis [10,11] and virosis (morbillivirus and herpesvirus (HV)) [12–18]. The number of publications concerning HV infection in cetaceans has been increased in the past decades. However, very little information is available regarding the presence of these viruses in BWs.

The *Herpesvirales* order is split into three families: *Alloherpes-*, *Herpes-*, and *Malacoherpesviridae*. The *Herpesviridae* family is divided into three subfamilies (*Alpha-*, *Beta-*, and *Gammaherpesvirinae*), 13 genera, and 107 species [International Committee on Taxonomy of Viruses (ICTV) (https://talk.ictvonline.org/taxonomy/ (accessed on 3 September 2020)) [19]. A single, linear, double-stranded DNA characterizes these viruses, which can cause immunosuppression [20] and latent infections [21,22]. Moreover, HV can infect a wide range of hosts (mammals, birds, reptiles, amphibians, fish, mollusks, and insects) [23].

In cetaceans, several pathological findings associated with HV infection (alpha- and gamma-herpesvirus) have been documented, although in some cases, HV-related lesions may not be present in the infected animals [24]. Specifically, alphaherpesvirus has been related to fatal systemic infections [25], lymphoid necrosis [17], interstitial nephritis [18] and encephalitis and meningoencephalitis [26–28]. Gammaherpesvirus, however, has been described as mainly associated with mucocutaneous, skin, and genital lesions [16,29–33], although it has been recently described the first detection of gammaherpesvirus in the central nervous system of several striped dolphins (*Stenella coeruleoalba*) stranded in the Cantabrian Sea, Spain [34].

Herpesviruses have been found in many cetacean species, which are summarized in Table 1.

Table 1. Summary of reported herpesvirus infection in cetaceans worldwide.

Species	Locality	AlphaHV	GammaHV
Phocoena phocoena	Sweden, Netherlands, Portugal Northern Hemisphere	[27,28,35]	[27,32]
Delphinapterus leucas	Canada	[36,37]	-
Tursiops truncatus	Atlantic coast of United States of America (USA) Spain: The Canary Islands Mediterranean Sea	[25,30,38]	[29,30,39]
Pseudorca crassidens	Pacific waters	[40]	-
Peponocephala electra	Pacific waters	[40]	-
Lagenorhynchus obliquidens	Pacific waters	[41]	-
Balaenoptera physalus	Mediterranean Sea	[42]	-
Stenella coeruleoalba	Portugal Spain: The Canary Islands Cantabrian Sea	[34,35]	[31,34,35]
Delphinus delphis	Portugal	[35]	[35]
Lagenorhynchus obscurus	South America	[43]	-
Stenella frontalis	South America	[44]	-
Sotalia guianensis	South America	[44]	[33]
Kogia sima	South America	[44]	-

Table 1. *Cont.*

Species	Locality	AlphaHV	GammaHV
Ziphius cavirostris	Spain: The Canary Islands	[17]	-
Mesoplodon densirostris	Spain: The Canary Islands Atlantic coast of United States of America (USA)	[18]	[16,30]
Grampus griseus	Atlantic coast of United States of America (USA)	-	[30]
Physeter macrocephalus	Japanese coast	-	[40]
Balaenoptera acutorostrata	Mediterranean Sea	-	[42]
Inia boliviensis	South America	-	[44]
Mesoplodon stejnegeri	Japanese coast	-	[40]

Note: (-): Non reported cases.

There are some documented cases of HV coinfection in cetaceans. Herpes- and morbillivirus co-infection have been reported in striped dolphins from the Mediterranean and Atlantic coasts [24,35,45] and from the Canary Islands [26]. Some cases of herpes- and papillomavirus coinfections have also been reported in Atlantic bottlenose dolphins from the Atlantic coast of the USA and Cuba [46,47].

To date, only seven HV-BW sequences, five alphaherpesviruses, and two gammaherpesviruses are available in GenBank. Concerning the alphaherpesviruses, most of them (3/5) have been detected in BWs stranded in the Canary Islands (two Cuvier's BW (GU066291 and KY680659) and one Blainville's BW (JN863234)). The only other BWs in which the alphaherpesvirus has been detected are three Cuvier's BWs, two stranded in the Mediterranean Sea in 2012 (KP995682 and KP995685) and the other stranded on the Atlantic coast of the Spanish mainland in 2015 (KY680659).

The present study aims to detect HVs (novel or already known) in samples from stranded BWs in the Canary Islands and to correlate this positivity with histopathology to identify lesions compatible with the HV infection. In addition, phylogenetic relationship between the obtained sequences and all the available ones, within the *Herpesviridae* family, detected in cetaceans will be performed.

2. Materials and Methods

In this study, 294 samples from 55 BWs stranded along the coasts of the Canary Islands, from November 1999 to May 2017, were analyzed. These BWs included 35 Cuvier's BWs and 20 specimens belonging to the *Mesoplodon* genus. This last group consists of 2 Sowerby's BWs (*Mesoplodon bidens*), 7 Blainville's BWs, 10 Gervais' BWs (*Mesoplodon europaeus*), and 1 True's BW (*Mesoplodon mirus*). Two of these animals have been already published previously (CET 243 and CET 294) [17,18]. Adults were more highly represented, while males and females were present in a similar proportion, in both groups of BWs. This information and other biological parameters (stranding epidemiology (type, location, and date) and life history data (species, age category, and sex)) are summarized in Tables 2 and 3. Five codes of conservation condition were established [48]: Code 1 (extremely fresh carcass, as an animal that has recently died or euthanized), Code 2 (fresh carcass), Code 3 (moderate decomposition), Code 4 (advanced decomposition), and Code 5 (mummified or skeletal remains).

Table 2. Biological, stranding conditions and analyzed samples for herpesvirus detection of the 35 *Ziphius cavirostris* specimens included in the present study.

ID CODE	SEX	AGE	SD	SL	SS	DC	TESTED SAMPLES
CET 86	F	A	27/11/1999	Tenerife	A	3	Skin, lung, liver, kidney
CET 103	M	J	19/04/2000	Fuerteventura	D	3	Lung, liver, kidney, brain
CET 108	F	A	10/06/2000	Tenerife	D	3	Skin, skeletal muscle, lung, liver, kidney
CET 113	F	S	16/07/2000	Tenerife	D	3	Skin, skeletal muscle
CET 181	M	S	24/09/2002	Fuerteventura	A	2	Skin, skeletal muscle, lung, mediastinal and mesenteric lymph node, liver, kidney, brain, spleen
CET 182	M	S	24/09/2002	Fuerteventura	D	2	Skin, skeletal muscle, lung, liver, mesenteric lymph node, kidney, brain, spleen
CET 183	M	S	24/09/2002	Fuerteventura	D	2	Skin, skeletal muscle, liver, mesenteric lymph node, kidney, brain
CET 184	M	S	24/09/2002	Fuerteventura	D	2	Skin, skeletal muscle, lung, liver, mediastinal and mesenteric lymph node, kidney, brain, spleen, thyroid
CET 189	F	A	27/09/2002	Fuerteventura	D	4	Skin, lung, liver, kidney
CET 236	F	C	21/03/2004	La Graciosa	D	3	Skin, skeletal muscle, lung, liver, kidney, brain
CET 264	F	N.D.	23/07/2004	Fuerteventura	D	4	Liver, skeletal muscle, lung, kidney
CET 265	M	A	24/07/2004	Fuerteventura	D	4	Skin, skeletal muscle, lung, liver, kidney
* CET 294	F	A	18/04/2005	Fuerteventura	D	4	Skin, skeletal muscle, lung, liver, spleen
CET 304	F	C	13/07/2005	Fuerteventura	D	2	Skin, skeletal muscle, lung, liver, kidney
CET 322	M	A	17/02/2006	Gran Canaria	D	4	Skin, lung, liver, kidney
CET 352	N.D.	J	06/07/2006	Tenerife	D	3	Lung, kidney, brain, spleen
CET 471	F	S	06/11/2008	Fuerteventura	D	2	Lung, kidney, brain, spleen
CET 503	F	A	21/09/2009	Gran Canaria	D	4	Lung, kidney
CET 576	F	A	16/05/2011	Lanzarote	D	2	Lung, kidney, brain, spleen
CET 579	M	S	13/06/2011	Tenerife	D	4	Lung, mesenteric lymph node, kidney, brain, spleen
CET 591	F	A	01/11/2011	Tenerife	D	4	Lung, prescapular lymph node, kidney, brain, spleen
CET 593	M	A	18/11/2011	Gran Canaria	D	4	Skin, lung, prescapular lymph node, liver, kidney
CET 620	M	A	20/05/2012	Gran Canaria	D	4	Skin, lung, liver, kidney, spleen
CET 624	F	A	13/07/2012	La Graciosa	D	3	Skin, lung, liver, mesenteric lymph node, kidney, brain
CET 645	M	J	09/02/2013	Lanzarote	D	4	Skin, liver, brain
CET 680	F	N	02/07/2013	Gran Canaria	D	4	Lung, intestine, mesenteric lymph node, kidney, brain, spleen
CET 688	F	A	18/11/2013	Gran Canaria	D	4	Brain
CET 712	F	S	28/04/2014	Fuerteventura	D	4	Prescapular lymph node, spleen
CET 719	F	A	06/06/2014	Lanzarote	D	3	Lung, mesenteric lymph node, kidney, spleen
CET 720	N.D.	S	10/06/2014	Fuerteventura	D	4	Lung, mesenteric lymph node, kidney, brain
CET 770	M	S	28/07/2015	Tenerife	D	3	Lung, intestine, mesenteric lymph node, brain, spleen
CET 771	F	A	05/08/2015	Tenerife	D	2	Lung, intestine, mesenteric lymph node, kidney, brain, spleen
CET 818	M	S	16/08/2016	Gran Canaria	D	4	Lung, intestine, mesenteric lymph node, kidney, brain, spleen
CET 833	N.D.	N.D.	13/02/2017	Tenerife	D	4	Lung, mesenteric lymph node, kidney, brain
CET 855	M	A	22/05/2017	Gran Canaria	D	3	Lung, intestine, mesenteric lymph node, kidney, brain, spleen

Remarks: (*): animal previously published; SD (stranding date); SL (stranding location); SS (stranding stage: A = alive; D = dead); DC (decomposition stage); SEX (F = female, M = male, ND = not determined); AGE (A = adult; S = subadult; J = juvenile; C = calf, N = neonate). DC (1 = extremely fresh carcass; 2 = fresh carcass, 3 = moderate decomposition, 4 = advanced decomposition, and 5 = mummified or skeletal remains).

Table 3. Biological, stranding conditions and analyzed samples for herpesvirus detection of the 20 animals belonging to the *Mesoplodon* genus included in the present study.

ID CODE	SPECIES	SEX	AGE	SD	SL	SS	DC	TESTED SAMPLES
CET 134	*M. europaeus*	F	C	28/06/2001	Gran Canaria	A	1	Skin, lung, liver, kidney, brain
CET 180	*M. densirostris*	F	A	24/09/2002	Fuerteventura	A	2	Skin
CET 185	*M. europaeus*	F	A	24/09/2002	Fuerteventura	D	2	Skin, skeletal muscle, lung, liver, mesenteric lymph node, kidney, brain, spleen
CET 213	*M. densirostris*	F	A	28/06/2003	Gran Canaria	A	1	Skin, skeletal muscle, lung, liver, kidney, brain
* CET 243	*M. densirostris*	M	A	18/04/2004	Tenerife	A	1	Skin, skeletal muscle, lung, liver, kidney
CET 259	*M. europaeus*	F	J	21/06/2004	Fuerteventura	D	2	Skin, skeletal muscle, lung, liver, kidney, brain
CET 333	*M. europaeus*	F	S	28/03/2006	El Hierro	A	2	Skin, skeletal muscle, lung, thymus, liver, mesenteric lymph node, kidney, brain, spleen
CET 334	*M. europaeus*	F	S	28/03/2006	El Hierro	A	2	Skin, skeletal muscle, lung, liver, kidney, brain, spleen
CET 338	*M. europaeus*	F	J	06/04/2006	Gran Canaria	D	2	Skin, skeletal muscle, lung, liver, blood, mesenteric lymph node, kidney, brain, spleen
CET 354	*M. europaeus*	M	C	28/07/2006	Tenerife	D	4	Skin, lung, liver, kidney, spleen
CET 379	*M. bidens*	M	A	16/04/2007	Lanzarote	D	2	Skin, lung, liver, kidney, brain, spleen
CET 510	*M. europaeus*	M	A	14/12/2009	Lanzarote	D	2	Skin, lung, liver, mesenteric lymph node, kidney, brain
CET 547	*M. europaeus*	M	A	29/08/2010	Fuerteventura	D	4	Skin, lung, liver, mesenteric lymph node, kidney, brain
CET 631	*M. europaeus*	M	A	21/10/2012	Fuerteventura	D	4	Skin, lung, penis, palate, esophagus, brain
CET 636	*M. mirus*	M	S	30/11/2012	El Hierro	D	2	Skin, lung, liver, mesenteric lymph node, kidney, brain, spleen
CET 695	*M. densirostris*	F	A	12/07/2014	Lanzarote	D	4	Skin, lung, liver, mesenteric lymph node, kidney, brain, spleen
CET 711	*M. densirostris*	M	S	03/04/2014	El Hierro	D	5	Skin, lung, liver, mesenteric lymph node, kidney, brain, spleen
CET 824	*M. densirostris*	F	A	11/11/2016	Fuerteventura	D	2	Skin, prescapular lymph node, liver, kidney, brain, spleen
CET 827	*M. bidens*	F	A	07/12/2016	La Gomera	A	4	Skin, lung, liver, mesenteric lymph node, intestine, brain, spleen
CET 852	*M. densirostris*	F	A	05/05/2017	Fuerteventura	D	2	Skin, lung, liver, mesenteric lymph node, kidney, brain, spleen

Notes: (*): animal previously published; SD (stranding date); SL (stranding location); SS (stranding stage: A = alive; D = dead); DC (decomposition stage); SEX (F = female, M = male), AGE (A = adult, S = subadult; J = juvenile; C = calf); SS (stranding stage: A = alive, D = dead); DC (1 = extremely fresh carcass, 2 = fresh carcass, 3 = moderate decomposition, 4 = advanced decomposition, and 5 = mummified or skeletal remains).

All the animals were submitted to a complete standardized necropsy [48,49], and representative tissue samples were collected for further analysis. For the histopathological study, collected samples were fixed in a 10% neutral buffered formalin solution, processed, and embedded in paraffin blocks, which were sectioned at 5 µm and stained with hematoxylin and eosin (HE). The slides were then visualized in an optical microscope with the objective to find HV-associated lesions. For molecular analysis, collected samples were frozen at −80 °C.

A wide range of tissue samples, according to availability in each case, were analyzed for the presence of HV DNA by PCR: lung (16.33%; (48/294)), kidney (15.65%; (46/294)), brain (12.93%; (38/294)), skin (12.59%, (37/294)), liver (12.24%; (36/294)), spleen (9.86%; (29/294)), mesenteric lymph node (8.16% (24/294)), skeletal muscle (6.12%; (18/294)), intestine (2.04%; (6/294)), prescapular lymph node (1.36%; (4/294)), mediastinal lymph node [0.68%; (2/294)], thyroid gland (0.34%; (1/294)), thymus (0.34%; (1/294)), palate (0.34%; (1/294)), and esophagus (0.34%; (1/294)). In addition, blood was analyzed in one animal (0.34%; (1/294)) (Tables 2 and 3).

Thawed samples were mechanically macerated in a lysis buffer and centrifuged. DNA/RNA extraction was simultaneously carried out from each 300 µL macerated sample by pressure filtration, by means of a QuickGene R Mini 80 nucleic acid isolation instrument, with the DNA Tissue Kit S (QuickGene, Kurabo, Japan) according to the manufacturer's instructions with some modifications: An RNA carrier (Applied BiosystemsTM, Thermo Fisher Scientific Waltham, MA, USA) was added during the lysis step, as previously published [50].

A panherpesvirus conventional nested polymerase chain reaction (PCR) was performed for HV detection, amplifying a fragment of the DNA polymerase gene of the *Herpesviridae* family of about 200 bp [51]. Two negative controls (non-template) for extraction and amplification and an amplification-positive control (known herpesvirus DNA previously obtained in our laboratory) were included in each protocol. Horizontal gel electrophoresis, in 5% agarose containing GelRed® (Biotium, Inc. California, USA), was performed for 5 µL of the obtained amplicons from the second PCR. Purification of PCR products was carried out using a Real Clean spin kit (REAL®, Durviz, s.l.,Valencia, Spain) to perform sequencing (the Sanger method).

Furthermore, a reverse transcription real-time polymerase chain reaction (RT-qPCR) based on SYBRN® Green dye (Bio-Rad Laboratories, Inc., California, CA, USA) [52] was performed for Cetacean morbillivirus (CeMV) detection only in those animals that were positive for HV.

The obtained HV sequences were compared with similar sequences retrieved from GenBank via a Blast search with the blastn algorithm (www.ncbi.nlm.nih.gov/blast/Blast.cgi/(accessed on 3 May 2021)) [53]. ClustalW was used to perform the HV multiple sequences alignment using MEGA X software (Pennsylvania, PA, USA) [54]. To construct the phylogenetic nucleotide tree, 73 alphaherpesvirus sequences were retrieved from GenBank. To root the phylogram, nine gammaherpesvirus sequences were used as the outgroup. The best substitution model for the nucleotide phylogenetic tree analysis was selected based on its lowest BIC score (Bayesian Information Criterion). Accordingly, a phylogenetic tree was constructed using the Maximum Likelihood Method and the Tamura 3-parameter with a discrete Gamma distribution to model the evolutionary rate differences among sites (5 categories (+G, parameter = 0.7779)). Bootstrap resampling (1000 replicates) was used to assess the reliability of the tree.

The nucleotide sequences were translated to the deduced amino acid sequences (70 alphaherpesvirus and 9 gammaherpesvirus sequences retrieved from GenBank). The deduced animo acid phylogenetic tree was built using the best substitution model based on its lowest BiC, a Maximum Likelihood Method and the Jones Taylor Thornton matrix-based model with a discrete Gamma distribution to model the evolutionary rate differences among sites (5 categories (+G, parameter = 17,401)). A bootstrap consensus tree for 1000 replicates was also performed.

A consensus tree was computed accepting the default 50% cut-off value (nodes supported by <50% of bootstrap replicates are collapsed), as previously proposed [55]. Only those bootstrap values equal or greater than 70% were considered valid among the remaining nodes (>50%). Furthermore, all sequences obtained from this study were submitted in GenBank, whose number accessions are from MZ066758 to MZ066765.

3. Results

Eight out of the fifty-five analyzed BWs (14.45%) had a positive result of the PCR test. Specifically, HV was detected in adult individuals of three *Ziphius cavirostris* and five animals belonging to the *Mesoplodon* genus (three *M. densirostris,* one *M. europaeus*, and one *M. bidens*), from which two had been previously published [17,18].

Moreover, these viruses were detected in 15 out of 294 (5.1%) analyzed samples. HV-positive samples were, by decreasing frequency, as follows: lungs (5/48, 10.41%), kidney (5/46, 10.89%), brain (2/38, 5.26%), liver (1/36, 2.77%), spleen (1/29, 3.44%), and prescapular lymph node (1/4, 25%).

3.1. Molecular Findings

Herpesvirus was detected in 3 out of 35 Cuvier's BWs (8.6%). Specifically, Cases 3, 5, and 8 were positive for HV in one or more tissues (Table 4) (Figure 1A). Case 3 (CET 294), an adult female in advanced decomposition, showed positivity for HV in spleen and lung samples [17]. In Case 5 (CET 771), an adult female in good state of preservation (code 2), HV was found in brain tissue; in Case 8 (CET 855), an adult male in moderate decomposition, the positivity was found in the lung. Thus, a total of 4 samples out of 168 (2.4%) were positive for HV in the *Ziphius cavirostris* group. By decreasing frequency, HV was detected in lung (2/30, 6.6%), brain (1/21, 4.8%), and spleen (1/17, 5.9%) samples (Table 4). The size of the new sequences obtained in the present study, (Cases 5 and 8) ranged from 210 to 234 bp.

Table 4. Beaked whales and samples positive for herpesvirus in our study.

CASE *n*°	ID CODE	SPECIES	SEX	AGE	SD	SL	SS	DC	HV-POSITIVE SAMPLES	HV-OBTAINED SEQUENCES
1	* CET 243	*M. de*	M	A	18/04/2002	T	A	1	Lung, kidney	(Arbelo et al., 2012)
2	CET 259	*M. eu*	F	A	21/06/2004	F	D	2	Kidney	MZ066758
3	* CET 294	*Z. ca*	F	A	18/04/2005	F	D	4	Lung, spleen	(Arbelo et al., 2010)
4	CET 379	*M. bi*	M	A	16/04/2007	L	D	2	Lung, kidney	MZ066759
5	CET 771	*Z. ca*	F	A	06/08/2015	T	D	2	Brain	MZ066760
6	CET 824	*M. de*	F	A	11/11/2016	F	D	2	(a) Liver, prescapular lymph node, kidney; (b) brain	(a) MZ066761, (b) MZ066762
7	CET 852	*M. de*	F	A	05/05/2017	F	D	2	(a) Lung; (b) kidney	(a) MZ066763, (b) MZ066764
8	CET 855	*Z. ca*	M	A	22/05/2017	GC	D	3	Lung	MZ066765

Remarks: (*): animal previously published. *M. de* (*Mesoplodon densirostris*); *M. eu* (*Mesoplodon europaeus*); *Z. ca* (*Ziphius cavirostris*); *M.bi* (*Mesoplodon bidens*); SEX (F = female, M = male); AGE (A = adult); SD (stranding date); SL (stranding location: T = Tenerife, F = Fuerteventura, L = Lanzarote, GC = Gran Canaria); SS (stranding stage: A = alive, D = dead); DC (decomposition stage: 1 = extremely fresh carcass, 2 = fresh carcass, 3 = moderate decomposition, 4 = advanced decomposition).

Five out of twenty animals (25%) were positive for HV within the *Mesoplodon* genus group (Cases 1, 2, 4, 6, and 7) in one or more tissues (Figure 1B–D). Specifically, a total of 11 samples out of 126 (8.7%) were positive for HV. By decreasing frequency, HV was detected in kidney (5/17, 29.4%), lung (3/18, 16.6%), liver (1/18, 5.5%), brain (1/17, 5.9%), and prescapular lymph node (1/1, 100%) samples (Table 4). HV was found in lung and kidney samples from Case 1 (CET 243), a very fresh (live stranded individual, which subsequently died) adult male of *M. densirostris* [18], Case 4 (CET 379), an adult male in code 2 of *M. bidens*, and Case 7 (CET 852), an adult female in code 2 of *M. densirostris*.

Herpesvirus was detected in just one tissue (kidney) in Case 2 (CET 259), an adult female in code 2 of *M. europaeus*. In Case 6 (CET 824), an adult male in code 2 of *M. bidens*, HV was found in liver, prescapular lymph node, kidney, and brain tissues. The size from the obtained six new sequences from our study (Cases 2, 4, 6, and 7) ranged from 194 to 234 bp. None of the HV-infected animals were positive for CeMV. Phylogenetic analysis showed that all the sequences obtained from both groups of BWs belonged to the *Alphaherpesvirinae* subfamily.

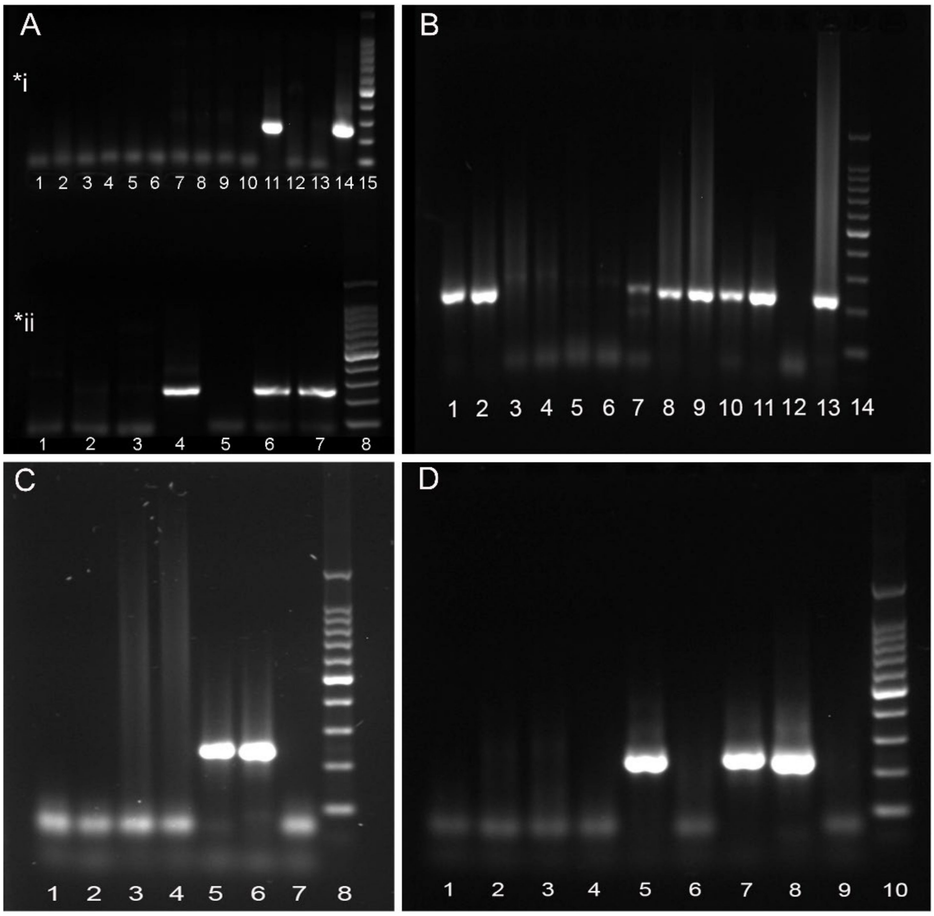


Figure 1. Herpesvirus-positive beaked whales results in agarose gel 5%, using nested conventional PCR. (**A**) (*i) Lane 11: Case 8 (CET 855—lung). Lane 14: PCR positive control. Lane 15: Molecular-weight size marker. (*ii) Lane 4: PCR positive control: Case 7 (CET 852—kidney). Lane 6 and 7: Case 5 (CET 771—brain). Lane 8: Molecular-weight size marker. (**B**) Lane 1: Case 4 (CET 379—lung). Lane 2: Case 4 (CET 379—kidney). Lane 8: Case 7 (CET 852—lung). Lane 9: Case 6 (CET 824—prescapular lymph node). Lane 10: Case 6 (CET 824—liver). Lane 11: Case 6 (CET 824—kidney). Lane 13: PCR positive control. Lane 14: Molecular-weight size marker. (**C**) Lane 5: Case 6 (CET 824—brain). Lane 6: PCR positive control. Lane 8: Molecular-weight size marker. (**D**) Lane 5: Case 2 (CET 259—kidney). Lane 7: Extraction positive control (CET 854—brain). Lane 8: PCR positive control/Case 7 (CET 852—kidney). Lane 10: Molecular-weight size marker. *Sequences from CET 243 and CET 294 have been already published.

3.1.1. Nucleotide Identity

Eight sequences obtained from this study were new; two were previously published [17,18]. Nucleotide similarities are summarized in Tables 5 and 6. The criteria to considerer a novel sequence are that the sequence are ≥100 bp long and has a <90% identity to the reference genome [56]. Based on this, the sequences from Cases 2 and 4 and the lung sample from Case 7 (7a) can be considered novel, as presented a 78.35%, 89.32% and 78.30%, similarity, respectively. Sequences from the kidney and lung in Case 1 (JN863234) were considered novel when they were published [18], although they currently show a high percentage of identity with a sequence detected in the brain of a striped dolphin stranded in the Mediterranean Sea in 2011. A novel sequence was obtained from the kidney in Case 2 (MZ066758); the sequence most closely related to this novel sequence was a previously published sequence detected in the prescapular lymph node of a Cuvier's BW stranded in the Mediterranean Sea in 2012. Sequencing and further comparison with GenBank records showed a novel sequence highly related to cetacean alphaherpesvirus in the lung and spleen samples from Case 3 (GU066291) when they were published [17]. However, these sequences are currently identical to four previously described sequences in striped dolphins. A novel sequence was amplified from the lung and kidney in Case 4 (MZ066759), showing the highest similarity with sequences amplified from the same organs in Case 1 from our study. Two different sequences were obtained in Case 6, one sequence from the liver, prescapular lymph node, and kidney (6a) (MZ066761) and another from the brain (6b) (MZ066761). Two different sequences were also obtained from Case 7, one from the lung (7a) (MZ066763) and other from the kidney (7b) (MZ066764). Sequence 7a is considered novel.

Table 5. Summary of the obtained sequences from *Ziphius cavirostris* in our study.

	*** Case 3**	**Case 5**	**Case 8**
CET	294	771	855
GenBank Acc. No.	GU066291	MZ066760	MZ066765
Samples	Lung and spleen	Brain	Lung
Nucleotide identity	100% MG437217 (*S. co*); KY680657 (*S.co*); KY680656 (*S. co*); KJ156331 (*S. co*)	98.48% KP995682 (*Z. ca*)	98.06% KP995682 (*Z. ca*)
Aminoacid identity	100% AUZ97325 (*S. co*) AUZ97326 (*S. co*) AHN91834 (*S. co*)	98.48% ALP00298 (*Z. ca*)	98.53% ALP00298 (*Z. ca*)

Notes: (*): animal previously published. *S. co* (*Stenella coeruleoalba*); *Z. ca* (*Ziphius cavirostris*).

Table 6. Summary of the obtained sequences from *the Mesoplodon genus* in our study. Bold percentages indicate novel sequences.

	*** Case 1**	**Case 2**	**Case 4**	**Case 6**	**Case 7**
CET	243	259	379	824	852
GenBank Acc. No.	JN863234	MZ066758	MZ066759	(a) MZ066761 (b) MZ066762	(a) MZ066763 (b) MZ066764
Species	*M. densirostris*	*M. europaeus*	*M. bidens*	*M. densirostris*	*M. densirostris*
Samples	Lung and kidney	Kidney	Lung and kidney	(a) Liver, prescapular lymph node and kidney (b) Brain	(a) Lung (b) Kidney
Nucleotide identity	95.65% KP995684 (*S.co*)	**78.35%** KP995682 (*Z. ca*)	**89.32%** JN863234 (*M. de*)	(a) 93.59% JN863234 (*M. de*) (b) 97.04% KP995684 (*S. co*)	(a) **78.3%** KF155406 (*D. le*) (b) 97.46% KP995684 (*S.co*)
Amino acid identity	79.13% ANG08598 (*D. le*)	82.81% ALP00298 (*Z. ca*)	95.59% ALP00300 (*S.co*)	(a) 97.06% ALP00300 (*S.co*) (b) 97.06% ALP00300 (*S.co*)	(a) 72.73% ALP00292 (*Z. ca*) (b) 96.92% ALP00300 (*S.co*)

Notes: (*): animal previously published. *S. co* (*Stenella coeruleoalba*); *D. le* (*Delphinapterus leucas*); *Z. ca* (*Ziphius cavirostris*); *M. de* (*Mesoplodon densirostris*).

The nucleotide phylogenetic analysis (Figure 2A) showed that sequences from Cases 1 (kidney), 4 (lung and kidney), 6a (liver, prescapular lymph node, and kidney), 6b (brain), and 8 (lung) clustered together in a clade supported by a bootstrap value of 64, with two subclades: one containing sequences from Cases 1, 4, 6a, and 6b and a sequence from a striped dolphin stranded in the Mediterranean Sea in 2011 (KP995684) (84 bootstrap value) and another containing two sequences, one from Case 8 and another from a Cuvier's BW stranded in the Mediterranean Sea in 2012 (KP995682) (79 bootstrap value).

The sequence from Case 7 (lung) did not cluster with any sequence within the nucleotide phylogenetic tree, nor did the sequence from Case 2 (kidney), even if both of them belong to a large clade (62 bootstrap value) containing all the cetaceans alphaherpesvirus published until now.

However, the sequence from Case 3 (lung and spleen) clustered with sequences detected in striped dolphins stranded in the Mediterranean and Atlantic coasts (92 bootstrap value); sequences from Cases 5 (brain) and 7 (kidney) clustered together (97 bootstrap value) and with a sequence (blowhole swab) from a beluga whale (MF678601) (100 bootstrap value).

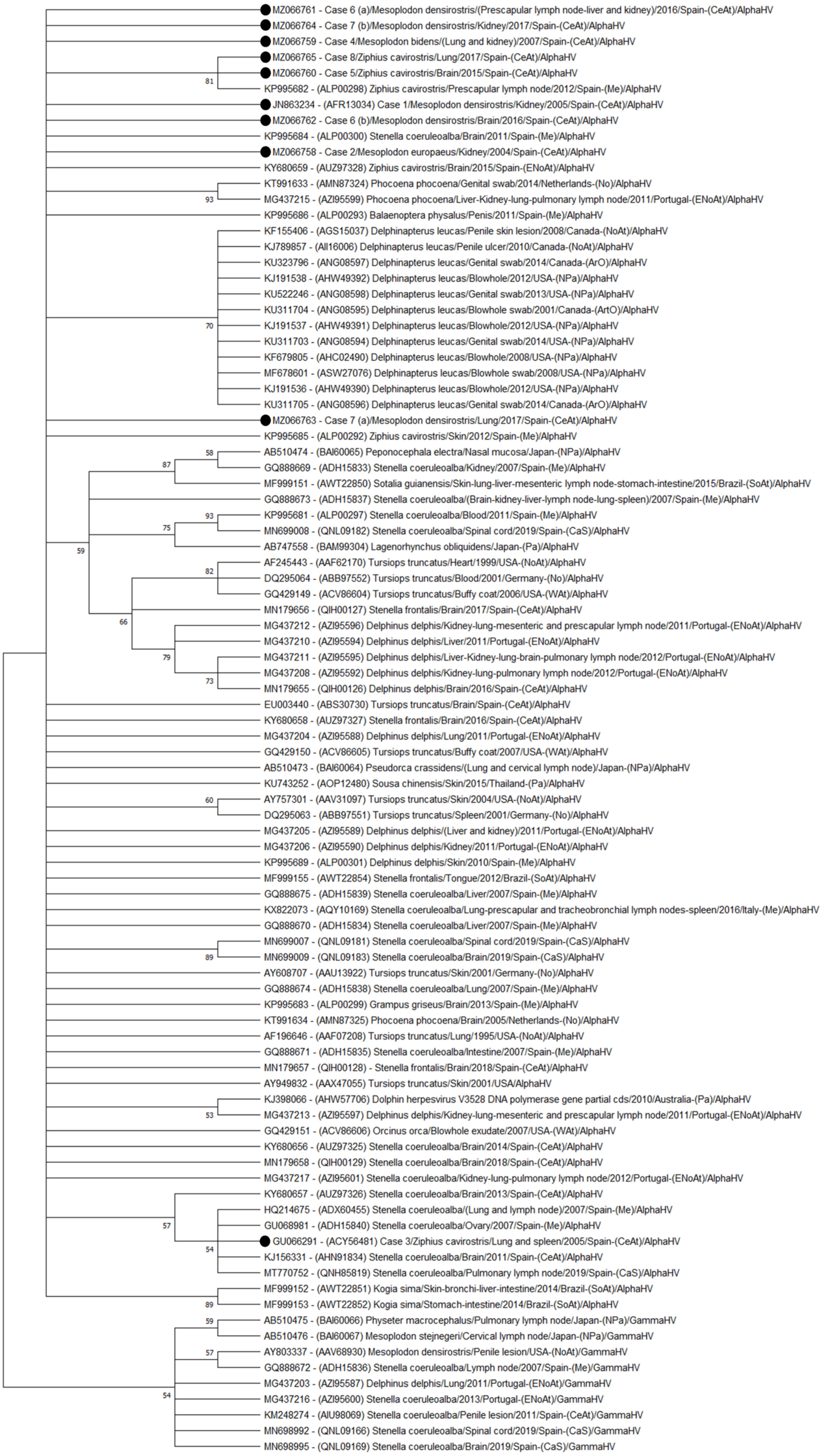
3.1.2. Amino Acid Identity

Blast analyses of translated amino acid sequences showed similar results to those of the nucleotides. However, some differences were observed for Cases 1, 4, 6a, and 7a. Case 1 showed the highest similarity (79.13%, 100 QC) with a sequence detected in a beluga whale (ANG08598). Sequences from the lung and kidney in Case 4 were very similar (95.59%, 87% QC) to a sequence detected in the brain of a striped dolphin stranded in the Mediterranean Sea in 2011 (ALP00300), and to a sequence detected in a Cuvier's BW stranded in the Mediterranean Sea in 2012 (ALP00298) (94.12%, 87% QC). The sequence from the prescapular lymph node, liver, and kidney in Case 6 (6a) was very similar (97.06%, 99 QC) to sequence ALP00300. The lung sequence from Case 7 showed the highest similarity (72.73%, 94% QC) with a sequence detected in the skin of a Cuvier's BW stranded in the Mediterranean Sea in 2012 (ALP00292). Amino acid similarities are summarized in Tables 5 and 6.

Phylogenetic analysis (Figure 2B) showed that the tree based on deduced amino acids consists of 45 alphaherpesvirus branches and a root that contains nine gammaherpesvirus sequences. All the obtained sequences from our study take part of a large polyphyletic clade within which seven sequences (Cases 1, 2, 4, 6a, 6b, 7a, and 7b) did not form sub-clades with any sequences of any of the previously identified HV in cetaceans; while sequences from Case 5 (brain) and Case 8 (lung) clustered together with a sequence obtained from the prescapular lymph node of a Cuvier's BW stranded in 2012 in the Mediterranean Sea (ALP00298) (bootstrap value of 81). Finally, the sequence from Case 3 clustered together with sequences obtained from striped dolphins stranded in the Mediterranean Sea and the Atlantic coasts of Spain (mainland and Canary Islands) and the Cantabrian Sea (54 bootstrap values).

(**A**)

Figure 2. *Cont*.


MZ066761 - Case 6 (a)/Mesoplodon densirostris/(Prescapular lymph node-liver and kidney)/2016/Spain-(CeAt)/AlphaHV
MZ066764 - Case 7 (b)/Mesoplodon densirostris/Kidney/2017/Spain-(CeAt)/AlphaHV
MZ066759 - Case 4/Mesoplodon bidens/(Lung and kidney)/2007/Spain-(CeAt)/AlphaHV
MZ066765 - Case 8/Ziphius cavirostris/Lung/2017/Spain-(CeAt)/AlphaHV
MZ066760 - Case 5/Ziphius cavirostris/Brain/2015/Spain-(CeAt)/AlphaHV
81
KP995682 - (ALP00298) Ziphius cavirostris/Prescapular lymph node/2012/Spain-(Me)/AlphaHV
JN863234 - (AFR13034) Case 1/Mesoplodon densirostris/Kidney/2005/Spain-(CeAt)/AlphaHV
MZ066762 - Case 6 (b)/Mesoplodon densirostris/Brain/2016/Spain-(CeAt)/AlphaHV
KP995684 - (ALP00300) Stenella coeruleoalba/Brain/2011/Spain-(Me)/AlphaHV
MZ066758 - Case 2/Mesoplodon europaeus/Kidney/2004/Spain-(CeAt)/AlphaHV
KY680659 - (AUZ97328) Ziphius cavirostris/Brain/2015/Spain-(ENoAt)/AlphaHV
KT991633 - (AMN87324) Phocoena phocoena/Genital swab/2014/Netherlands-(No)/AlphaHV
93
MG437215 - (AZI95599) Phocoena phocoena/Liver-Kidney-lung-pulmonary lymph node/2011/Portugal-(ENoAt)/AlphaHV
KP995686 - (ALP00293) Balaenoptera physalus/Penis/2011/Spain-(Me)/AlphaHV
KF155406 - (AGS15037) Delphinapterus leucas/Penile skin lesion/2008/Canada-(NoAt)/AlphaHV
KJ789857 - (AII16006) Delphinapterus leucas/Penile ulcer/2010/Canada-(NoAt)/AlphaHV
KU323796 - (ANG08597) Delphinapterus leucas/Genital swab/2014/Canada-(ArO)/AlphaHV
KJ191538 - (AHW49392) Delphinapterus leucas/Blowhole/2012/USA-(NPa)/AlphaHV
KU522246 - (ANG08598) Delphinapterus leucas/Genital swab/2013/USA-(NPa)/AlphaHV
KU311704 - (ANG08595) Delphinapterus leucas/Blowhole swab/2001/Canada-(ArtO)/AlphaHV
70
KJ191537 - (AHW49391) Delphinapterus leucas/Blowhole/2012/USA-(NPa)/AlphaHV
KU311703 - (ANG08594) Delphinapterus leucas/Genital swab/2014/USA-(NPa)/AlphaHV
KF679805 - (AHC02490) Delphinapterus leucas/Blowhole/2008/USA-(NPa)/AlphaHV
MF678601 - (ASW27076) Delphinapterus leucas/Blowhole swab/2008/USA-(NPa)/AlphaHV
KJ191536 - (AHW49390) Delphinapterus leucas/Blowhole/2012/USA-(NPa)/AlphaHV
KU311705 - (ANG08596) Delphinapterus leucas/Genital swab/2014/Canada-(ArO)/AlphaHV
MZ066763 - Case 7 (a)/Mesoplodon densirostris/Lung/2017/Spain-(CeAt)/AlphaHV
KP995685 - (ALP00292) Ziphius cavirostris/Skin/2012/Spain-(Me)/AlphaHV
58
AB510474 - (BAI60065) Peponocephala electra/Nasal mucosa/Japan-(NPa)/AlphaHV
87
GQ888669 - (ADH15833) Stenella coeruleoalba/Kidney/2007/Spain-(Me)/AlphaHV
MF999151 - (AWT22850) Sotalia guianensis/Skin-lung-liver-mesenteric lymph node-stomach-intestine/2015/Brazil-(SoAt)/AlphaHV
GQ888673 - (ADH15837) Stenella coeruleoalba/(Brain-kidney-liver-lymph node-lung-spleen)/2007/Spain-(Me)/AlphaHV
93
KP995681 - (ALP00297) Stenella coeruleoalba/Blood/2011/Spain-(Me)/AlphaHV
75
MN699008 - (QNL09182) Stenella coeruleoalba/Spinal cord/2019/Spain-(CaS)/AlphaHV
59
AB747558 - (BAM99304) Lagenorhynchus obliquidens/Japan-(Pa)/AlphaHV
AF245443 - (AAF62170) Tursiops truncatus/Heart/1999/USA-(NoAt)/AlphaHV
82
DQ295064 - (ABB97552) Tursiops truncatus/Blood/2001/Germany-(No)/AlphaHV
GQ429149 - (ACV86604) Tursiops truncatus/Buffy coat/2006/USA-(WAt)/AlphaHV
MN179656 - (QIH00127) Stenella frontalis/Brain/2017/Spain-(CeAt)/AlphaHV
66
MG437212 - (AZI95596) Delphinus delphis/Kidney-lung-mesenteric and prescapular lymph node/2011/Portugal-(ENoAt)/AlphaHV
MG437210 - (AZI95594) Delphinus delphis/Liver/2011/Portugal-(ENoAt)/AlphaHV
79
MG437211 - (AZI95595) Delphinus delphis/Liver-Kidney-lung-brain-pulmonary lymph node/2012/Portugal-(ENoAt)/AlphaHV
MG437208 - (AZI95592) Delphinus delphis/Kidney-lung-pulmonary lymph node/2012/Portugal-(ENoAt)/AlphaHV
73
MN179655 - (QIH00126) Delphinus delphis/Brain/2016/Spain-(CeAt)/AlphaHV
EU003440 - (ABS30730) Tursiops truncatus/Brain/Spain-(CeAt)/AlphaHV
KY680658 - (AUZ97327) Stenella frontalis/Brain/2016/Spain-(CeAt)/AlphaHV
MG437204 - (AZI95588) Delphinus delphis/Lung/2011/Portugal-(ENoAt)/AlphaHV
GQ429150 - (ACV86605) Tursiops truncatus/Buffy coat/2007/USA-(WAt)/AlphaHV
AB510473 - (BAI60064) Pseudorca crassidens/(Lung and cervical lymph node)/Japan-(NPa)/AlphaHV
KU743252 - (AOP12480) Sousa chinensis/Skin/2015/Thailand-(Pa)/AlphaHV
60
AY757301 - (AAV31097) Tursiops truncatus/Skin/2004/USA-(NoAt)/AlphaHV
DQ295063 - (ABB97551) Tursiops truncatus/Spleen/2001/Germany-(No)/AlphaHV
MG437205 - (AZI95589) Delphinus delphis/(Liver and kidney)/2011/Portugal-(ENoAt)/AlphaHV
MG437206 - (AZI95590) Delphinus delphis/Kidney/2011/Portugal-(ENoAt)/AlphaHV
KP995689 - (ALP00301) Delphinus delphis/Skin/2010/Spain-(Me)/AlphaHV
MF999155 - (AWT22854) Stenella frontalis/Tongue/2012/Brazil-(SoAt)/AlphaHV
GQ888675 - (ADH15839) Stenella coeruleoalba/Liver/2007/Spain-(Me)/AlphaHV
KX822073 - (AQY10169) Stenella coeruleoalba/Lung-prescapular and tracheobronchial lymph nodes-spleen/2016/Italy-(Me)/AlphaHV
GQ888670 - (ADH15834) Stenella coeruleoalba/Liver/2007/Spain-(Me)/AlphaHV
MN699007 - (QNL09181) Stenella coeruleoalba/Spinal cord/2019/Spain-(CaS)/AlphaHV
89
MN699009 - (QNL09183) Stenella coeruleoalba/Brain/2019/Spain-(CaS)/AlphaHV
AY608707 - (AAU13922) Tursiops truncatus/Skin/2001/Germany-(No)/AlphaHV
GQ888674 - (ADH15838) Stenella coeruleoalba/Lung/2007/Spain-(Me)/AlphaHV
KP995683 - (ALP00299) Grampus griseus/Brain/2013/Spain-(Me)/AlphaHV
KT991634 - (AMN87325) Phocoena phocoena/Brain/2005/Netherlands-(No)/AlphaHV
AF196646 - (AAF07208) Tursiops truncatus/Lung/1995/USA-(NoAt)/AlphaHV
GQ888671 - (ADH15835) Stenella coeruleoalba/Intestine/2007/Spain-(Me)/AlphaHV
MN179657 - (QIH00128) - Stenella frontalis/Brain/2018/Spain-(CeAt)/AlphaHV
AY949832 - (AAX47055) Tursiops truncatus/Skin/2001/USA/AlphaHV
KJ398066 - (AHW57706) Dolphin herpesvirus V3528 DNA polymerase gene partial cds/2010/Australia-(Pa)/AlphaHV
53
MG437213 - (AZI95597) Delphinus delphis/Kidney-lung-mesenteric and prescapular lymph node/2011/Portugal-(ENoAt)/AlphaHV
GQ429151 - (ACV86606) Orcinus orca/Blowhole exudate/2007/USA-(WAt)/AlphaHV
KY680656 - (AUZ97325) Stenella coeruleoalba/Brain/2014/Spain-(CeAt)/AlphaHV
MN179658 - (QIH00129) Stenella coeruleoalba/Brain/2018/Spain-(CeAt)/AlphaHV
MG437217 - (AZI95601) Stenella coeruleoalba/Kidney-lung-pulmonary lymph node/2012/Portugal-(ENoAt)/AlphaHV
KY680657 - (AUZ97326) Stenella coeruleoalba/Brain/2013/Spain-(CeAt)/AlphaHV
HQ214675 - (ADX60455) Stenella coeruleoalba/(Lung and lymph node)/2007/Spain-(Me)/AlphaHV
57
GU068981 - (ADH15840) Stenella coeruleoalba/Ovary/2007/Spain-(Me)/AlphaHV
GU066291 - (ACY56481) Case 3/Ziphius cavirostris/Lung and spleen/2005/Spain-(CeAt)/AlphaHV
54
KJ156331 - (AHN91834) Stenella coeruleoalba/Brain/2011/Spain-(CeAt)/AlphaHV
MT770752 - (QNH85819) Stenella coeruleoalba/Pulmonary lymph node/2019/Spain-(CaS)/AlphaHV
MF999152 - (AWT22851) Kogia sima/Skin-bronchi-liver-intestine/2014/Brazil-(SoAt)/AlphaHV
89
MF999153 - (AWT22852) Kogia sima/Stomach-intestine/2014/Brazil-(SoAt)/AlphaHV
59
AB510475 - (BAI60066) Physeter macrocephalus/Pulmonary lymph node/Japan-(NPa)/GammaHV
AB510476 - (BAI60067) Mesoplodon stejnegeri/Cervical lymph node/Japan-(NPa)/GammaHV
57
AY803337 - (AAV68930) Mesoplodon densirostris/Penile lesion/USA-(NoAt)/GammaHV
GQ888672 - (ADH15836) Stenella coeruleoalba/Lymph node/2007/Spain-(Me)/GammaHV
MG437203 - (AZI95587) Delphinus delphis/Lung/2011/Portugal-(ENoAt)/GammaHV
54
MG437216 - (AZI95600) Stenella coeruleoalba/2013/Portugal-(ENoAt)/GammaHV
KM248274 - (AIU98069) Stenella coeruleoalba/Penile lesion/2011/Spain-(CeAt)/GammaHV
MN698992 - (QNL09166) Stenella coeruleoalba/Spinal cord/2019/Spain-(CaS)/GammaHV
MN698995 - (QNL09169) Stenella coeruleoalba/Brain/2019/Spain-(CaS)/GammaHV

(**B**)

Figure 2. Maximum Likelihood phylogenetic trees. Nucleotide and amino acid sequences were identified with their corresponding accession number from GenBank, the host, the sample of detection, the date of collection, and the geographic area of stranding. Abbreviations: NoAt (North Atlantic Ocean); ENoAt (Northeast Atlantic Ocean); WAt (West Atlantic Ocean); CeAt (Central Atlantic Ocean); SoAt (South Atlantic Ocean); Me (Mediterranean Sea); CaS (Cantabrian Sea); Pa (Pacific Ocean); NPa (North Pacific Ocean); No (North Sea); ArO (Arctic Ocean). The bootstrap analysis was made to resample 1000 replicates and evaluate the reliability of the both trees. (**A**) Molecular phylogenetic analysis based on 94 nucleotide sequences from the polymerase gene of cetacean alphaherpesvirus. The Neighbor-Join and BioNJ algorithms along with the Tamura 3-parameter model and Gamma distribution were used to construct the tree. (**B**) Molecular phylogenetic analysis based on 92 amino acid sequences from the polymerase gene of cetacean alphaherpesviruses. The Neighbor-Join and BioNJ algorithms along with the JTT model and Gamma distribution were used to construct the tree.

3.2. Gross and Histopathological Findings

None of the HV-positive animals showed gross lesions associated with Herpesvirus infection, except Case 7, which presented several ulcerative and well-defined round skin lesions.

At the histopathological level, three animals presented lesions attributable to HV infection, one within the *Ziphius cavirostris* group and two within the *Mesoplodon* genus group. Specifically, Case 3 (*Z. cavirostris*) presented diffuse lymphoid and splenic necrosis with intranuclear inclusion bodies in monocytes [17]; Case 1 (*M. densirostris*) [18] and Case 7 (*M. densirostris*) displayed similar lesions, characterized by membranous glomerulonephritis and lymphoplasmacytic interstitial nephritis at the cortico-medullary region, moderate multifocal interstitial and tubuloepithelial necrosis with the presence of intranuclear inclusion bodies within tubuloepithelial cells in the renal medulla; mainly in the blood capillary network defined as the vasa recta of the kidney (*vasa rectae renis*) (Figure 3).

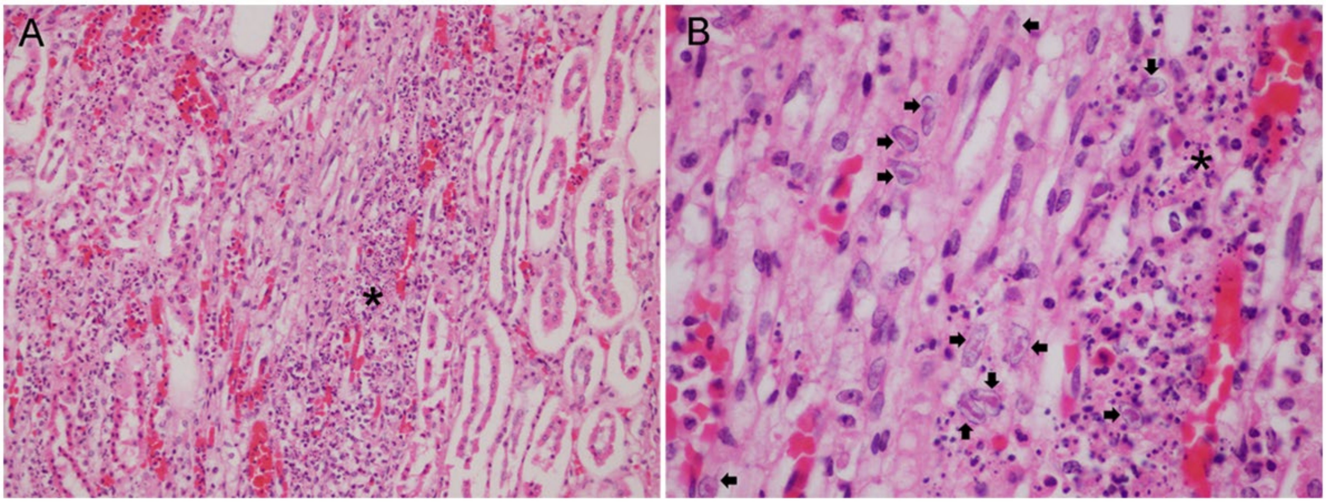


Figure 3. Histopathological findings from the kidney in Case 8 (CET 852). (**A**) Moderate and multifocal acute tubular necrosis in the medullary zone (asterisk). Hematoxylin and eosin, X20. (**B**) A moderate and multifocal presence of intranuclear inclusion bodies (arrows) associated to acute tubular necrosis (asterisk). Hematoxylin and eosin, X60.

Additional findings in the other positive HV-BWs were as follows: mild multifocal parasitic bronchopneumonia with the presence of intraluminal nematodes in Cases 1 and 4; generalized lymphadenopathy and lymphoplasmacytic interstitial nephritis in Case 4; severe parasitic nephritis and verminous mesenteric arteritis by *Crassicauda* sp., a moderate multifocal suppurative bronchopneumonia, and moderate multifocal suppurative lymphadenitis in Case 5; periductal fibrosis with lymphoplasmacytic pericolangitis in the liver, as a result of severe parasitic infection, in Case 6; the presence of severe hyaline membranes and mild multifocal interstitial bronchopneumonia in Case 8.

In addition, Case 2 also displayed several skin lacerations in the caudal peduncle produced by fishing tackles and diffuse congestion and hemorrhages in the lungs, liver, kidneys, and adrenal glands, and Case 6 showed active stranding related lesions, consistent with skin lacerations, a hypercontraction of muscle fibers, and hyaline globules within hepatocyte cells.

4. Discussion

This study represents the first systematic survey of HV infection in cetaceans from the *Ziphiidae* family. Beaked whales are found in all oceans and are of particular interest because they are one of the deepest diving whales and there is a proven relationship between several mass stranding events of BWs and military operations employing mid-frequency sonar [1,7,8].

We have detected HV in 14.45% (*n* = 8) of the surveyed BWs (*n* = 55). A percentage positivity of 8.57% (3/35) was found within Cuvier's BW group, while the percentage of positivity rose to 25% (5/20) within the *Mesoplodon* group. Previously published prevalence of HV infection in stranded cetaceans include: 5.3% in the Western North Pacific (Japan) [40], 3.7% in Brazil [44], 7.8% in Portugal [35], and 41.2% in Cantabria, Spain [34]. Most of the references of HV infection in BWs are case reports: two alphaherpesviruses in a Cuvier's BW and a Blainville's BW stranded in the Canary Islands [17,18] and one gammaherpesvirus in a Blainville's BW stranded in the USA [16]. The detection of a gammaherpesvirus in a Stejneger's BW from the Japanese coasts took part of a survey of 76 stranded cetaceans that include 4 BWs (one Stejneger's BW and three Blainville's BWs) [40]. In addition, three sequences from the brain, skin, and prescapular lymph nodes of Cuvier's BWs are available in GenBank (KY680659, KP995685, and KP995682).

All the sequences obtained from this study belonged to the *Alphaherpesvirinae* subfamily in contrast to similar previous studies, in which both alpha and gammaherpesvirus were detected [27,34,35,40,44].

The *Ziphiidae* includes 22 species in 6 genera, being the second largest family of cetaceans after the *Delphinidae* [57]. Herpesvirus sequences detected in BWs from our study should be then analyzed by species. Specifically, sequences from Cuvier's BWs are not novel sequences, displaying higher homologies (100–98.06%) with sequences from striped dolphins stranded in the Mediterranean Sea and central and northeast Atlantic coasts (the Canary Islands and Portugal) (MG437217, KY680657, KY680656, and KJ156331) [26,34,35] (Case 1) and with a sequence detected in other Cuvier's BW from the Mediterranean Sea (KP995682) (Cases 5 and 8). The sequence KP995682 can be considered novel since it shows the highest homology (83.98%) with the sequence detected in the brain of a striped dolphin stranded in the Mediterranean Sea in 2011 (KP995684), which in turn showed the highest homology with the sequence from Case 1 in our study (95.5%) and with the sequence KP995682 (83.98%). This relationship could indicate that HV transmission has occurred between these proximal regions, as previously suggested for other alphaherpesviruses [26,35,44] and between these two different species. Cuvier's BWs are found in most oceans and seas worldwide (in temperate, subtropical, and tropical waters), and have the most extensive range of all BWs species, although the seasonality and migration patterns of this species are still unknown [58].

Regarding the *Mesoplodon genus*, sequences from the *M. densirostris* species are, in general, highly (97.4–93%) related to a sequence detected in the brain of a striped dolphin stranded in the Mediterranean Sea in 2011 (KP995684) (Cases 1, 6b, and 7b). As we mentioned before, this sequence, even if detected in a striped dolphin, showed its highest homologies with sequences detected in members of the *Ziphiidae* family, suggesting the idea of HV transmission from BWs to the striped dolphin species rather than the contrary. The only sequence from Case 7a is considered novel within the *M. densirostris* species in our study, being related to the sequence obtained from the penile skin lesion of a beluga whale stranded in St. Lawrence Estuary (Canada) (KF155406). This beluga sequence was also considered novel and tentatively named beluga whale herpesvirus [36]. The relationship (geographical and interspecies) between these two sequences is, for the moment, unknown. Blainville's BWs are little-known members of the *Ziphiidae*, living in tropical to temperate waters worldwide. There is little information on the abundance of Blainville's BWs worldwide, although they are considered to have the most extensive distribution of any whale in the *Mesoplodon* genus [59].

Two novel sequences were obtained from *M. europaeus* (Case 2) and *M. bidens* (Case 4) species. Sequence from Case 2 was related to sequence KP995682, detected in a Cuvier's BW from the Mediterranean Sea; as most of the Cuvier's BWs from our study; while sequence from Case 4 was related to sequence from Case 1 (JN863234), detected in a Blainville's BW stranded in the Canaries and taking part of this study. Interspecific interactions between these four species of BWs could explain HV transmission within the *Ziphiidae* family. Gervais's and Sowerby's BWs are little-known members of the *Ziphiidae*. Both species of BWs are distributed throughout the Atlantic Ocean, although it is unknown if they undertake seasonal movements or migrations [60,61]. The presence of both BWs species in the Canary archipelago is reported as sporadic [62,63].

Regarding the nucleotide phylogeny of the reported HV-BW sequences from our study (n = 10), most of the sequences (80%, 8/10) clustered with a sequence previously identified in a Cuvier's BW, but also from other species, such as striped dolphins or beluga whales. Specifically, the sequence from the lung in Case 7 (7a) clustered with the sequence from Case 5 and with a sequence detected in the blowhole swab of a beluga whale (MF678601) in the nucleotide tree. The phylogenetic analyses showed that the virus isolated from this beluga whale grouped with members of the genus *Varicellovirus*, in the subfamily *Alphaherpesvirinae*, and it was tentatively named Monodontid alphaherpesvirus 1 [64]. Thus, although most of the HV-BWs sequences from our study are quite host-specific, as previously suggested for the members of *Herpesviridae* [65], it seems that there is a possible interspecific transfer of these viruses. The obtained results from the amino acid phylogenetic analysis showed, however, that sequences from animals within the *Mesoplodon* genus group (70%, 7/10) did not cluster with any previously identified sequence. This discrepancy between trees could be due to the presence of too few characters in the aminoacid dataset, being the phylogenetic signal for the tree reconstruction, in accordance, too small. Sequences from the Cuvier's BW group (n = 3) clustered with a sequence previously detected in a Cuvier's BW (20%, 2/10) or a striped dolphin (10%, 1/10). However, apart from this study, there are no alphaherpesvirus sequences from animals within the *Mesoplodon* genus available in GenBank. More and larger sequences will be needed to better understand the species specificity of HV-BWs. In conclusion, from the phylogeny analyses, it can be observed that the obtained BW sequences from this study are more often closely related to each other and occasionally with sequences from other cetacean species, specifically striped dolphins and beluga whales. However, the short partial sequences of the catalytic subunit (UL30) of the DNA polymerase available from this and other studies, only allow for subfamily identification [66]. Further studies are needed to better understand the phylogenetic relationship between these and other BW sequences within these species.

In addition, to our knowledge, this is the first report of HV infection in two species of BWs: Gervais' and Sowerby's BWs, respectively.

Two different sequences were obtained within the same animal in Cases 6 and 7. Coinfection with different viral strains seems to be a common feature of HV infection in cetaceans as it has been previously reported in several studies [24,26,30,35].

Most HV-BWs in our study were detected in the lung and/or kidney, representing a percentage of 21.3% considering all the analyzed samples. However, if we consider only positive samples, this percentage increased to 66.7%. Moreover, HV was detected in the lung in 62% of the positive BWs (5/8) and the kidney in the same proportion. However, if we consider the two groups of BWs, HV was detected in the kidney in 100% of HV-positive animals within the *Mesoplodon* group of our study. A minor proportion of samples were positive for HV, specifically the brain, prescapular lymph node, liver, and spleen, being the first identification of an HV DNA sequence in a liver sample from a BW.

The capacity of HV to cause disease is uncertain displaying the broad pathogenic and epidemiological features of the disease. In cetaceans, there have been descriptions from asymptomatic cases of HV infections to systemic and/or central nervous system infections. No gross or histopathological lesions attributable to the virus infection were observed in most of the positive samples in our study (62.5%). In addition, despite the presence of skin lesions compatible with herpesvirus infection in Case 7, no HV DNA was detected in the corresponding sample. All the HV-infected BWs from our study were adults, although only three out of eight (37.5%) displayed histopathological lesions indicative of active herpesviral replication, consistent with previous publications [17,18]. It is well known that the role of the viral factors in the course of a infection is both determined by viral and host factors, including the immune status itself or in combination with the age of the individuals [67,68]. In this study, we described multifocal interstitial nephritis, tubuloepithelial necrosis, and the presence of several intranuclear inclusions in Case 7, lesions that are very similar to those previously described in other *M. densirostris* stranded in the Canary Islands 13 years earlier [18]. The animal from Case 3 (CET 294) presented an advance stage of decomposition (code 4), which could partially impair DNA integrity for PCR detection, as previously published [35]. However, HV was detected in lung and spleen samples, which showed severe histopathological lesions and large number of intranuclear inclusion bodies indicative of early stages of the infection and high viral load, allowing molecular detection and identification of the virus. In a similar way, HVs were detected in code 4 cetaceans, specifically two mysticeti species stranded in the Mediterranean Sea [42] and in an Atlantic spotted dolphin stranded in Brazil [44].

5. Conclusions

This research describes the presence of HV in BWs stranded in the Canary Islands over a 19-year period (1999–2017) by molecular methods. Our results showed a prevalence of positive BWs of 14.45% (8/55), representing the first systematic survey of this pathogen in BWs. Three out of eight HV-positive BWs displayed histopathological lesions indicative of active viral replication, which is in concordance with the latent period of most herpesviruses. However, HVs are also capable of causing severe disease in association with other pathogens, such as CeMV. No CeMV infection was detected in any of the HV-positive BWs, highlighting the potential disease-causing capacity of these viruses as primary pathogens. Eight new HV sequences were detected in this study, which were analyzed and compared to all HV existing sequences in cetaceans. Most of these sequences did not cluster with any other sequences in the amino acid phylogenetic trees, indicating a possible species-specificity in BWs; although testing for clustering and host specificity would need more tailored analyses. In addition, three novel sequences of a partial fragment of the conserved DNA polymerase of HVs are described, all of them within the *Mesoplodon* genus group. To our knowledge, this work is the first to describe herpesvirus infection in two species of BWs: Gervais' and Sowerby's BWs.

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Data Availability Statement: All beaked whales sequences have been deposited in GenBank (accession numbers: MZ066758, MZ066759, MZ066760, MZ066761, MZ066762, MZ066763, MZ066764, and MZ066765).

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5.2. ARTÍCULO 2:

Molecular Diagnosis of *Cetacean Morbillivirus* in Beaked Whales Stranded in the Canary Islands (1999–2017).

veterinary sciences

MDPI

Article

Molecular Diagnosis of Cetacean Morbillivirus in Beaked Whales Stranded in the Canary Islands (1999–2017)

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Abstract: A retrospective survey for detecting the cetacean morbillivirus (CeMV) was carried out in beaked whales (BWs) stranded in the Canary Islands (1999–2017). CeMV is responsible for causing worldwide epizootic events with the highest mass die-offs in cetaceans, although the epidemic status of the Canarian Archipelago seems to be that of an endemic situation. A total of 319 tissue samples from 55 BWs (35 Cuvier's BWs and 20 specimens belonging to the *Mesoplodon* genus) were subjected to the amplification of a fragment of the fusion protein (F) and/or phosphoprotein (P) genes of CeMV by means of one or more of three polymerase chain reactions (PCR). RNA integrity could not be demonstrated in samples from 11 animals. Positivity (dolphin morbillivirus strain (DMV)) was detected in the skin sample of only a subadult male Cuvier's BW stranded in 2002, being the earliest confirmed occurrence of DMV in the Cuvier's BW species. The obtained P gene sequence showed the closest relationship with other DMVs detected in a striped dolphin stranded in the Canary Islands in the same year. A phylogenetic analysis supports a previous hypothesis of a cross-species infection and the existence of the circulation of endemic DMV strains in the Atlantic Ocean similar to those later detected in the North-East Atlantic, the Mediterranean Sea and the South-West Pacific.

Keywords: cetaceans; morbillivirus; beaked whales; Canary Islands; PCR; *Ziphius cavirostris*; *Ziphiidae*

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1. Introduction

The cetacean morbillivirus (CeMV; genus *Morbillivirus*, *Paramyxoviridae* family, order *Mononegavirales*), consisting of a single linear molecule of negative-sense single-stranded RNA, has been responsible for major epizootic diseases in cetaceans, causing many of the biggest mass die-offs worldwide in these species [1]. The main pathological findings described in infected cetaceans are broncho-interstitial pneumonia, lymphoid depletion and nonsuppurative meningoencephalitis, as well as an increased susceptibility to opportunistic infections [1–5]. According to the stage of the infection, four presentation forms of the disease have been recently described [1]: acute and subacute systemic diseases, chronic systemic infections and chronic localized CeMV encephalitis.

Two lineages of CeMV have been proposed [1,6]: CeMV-1 for the "old" northern hemisphere lineage that includes dolphin morbillivirus (DMV) [7], porpoise morbillivirus (PMV) [7], pilot-whale morbillivirus (PWMV) [8] and beaked-whale morbillivirus (BWMV) [9] strains; and CeMV-2 for the "new" southern hemisphere lineage, consisting of virus strains detected in a Guiana dolphin (*Sotalia guianensis*) from Brazil [10] and in Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) from Western Australia [11]. In addition, it has been recently reported that a novel morbillivirus was detected in a Fraser's dolphin (*Lagenodelphis hosei*) stranded in Hawaii that is dissimilar to the BWMV previously identified from Hawaii and to other CeMV strains, showing an 83.9–88.7% nucleotide similarity depending on the P or N gens of these reported sequences [12].

Few cases of CeMV infections have been previously reported in beaked whales (BWs). The infection (BWMV strain) was first documented in a Longman's BW (*Indopacetus pacificus*) stranded in Hawaii in 2010 [9,13]. A lymphoplasmacytic (nonsuppurative) primarily cerebral encephalitis co-infection with the herpesvirus was observed in that case. The same strain has also been detected in Hawaii in two BWs; in a Cuvier's BW (*Ziphius cavirostris*) and in a Blainville's BW (*Mesoplodon densirostris*) stranded in 2008 and 2010, respectively [13]. Pathological descriptions were not available for those two cases. The infection (DMV strain) has also been recognized in one of the seven Longman's BWs that stranded together in Southern New Caledonia in 2013 [14], with no pathological descriptions; and in a Cuvier's BW stranded in Italy in 2015 [15] with mild pathological findings in its lungs. More details (multifocal fibrinous bronco-pneumonia with mild, multifocal necrotizing bronchiolitis) were revealed upon a microscopic examination and associated with verocytotoxic (VT1) *Escherichia coli*.

Beaked whales are deep-diving marine mammals, a condition that predisposes them to suffer from decompression sickness (DCS) associated with the employ of mid-frequency sonar during military operations [16–23]. Several mass stranding events of BWs have occurred in the Canary Islands related to naval exercises, although there have been no more since the Spanish government imposed a moratorium on naval exercises in these waters in 2004 [24,25]. However, these species also face other anthropogenic threats, such as entanglements in or ingestions of marine litter and ship strikes [26–28]. They are also vulnerable to infectious diseases, such as in the described cases of verminous arteritis by *Crassicauda* spp. [29], brucellosis [30,31], the herpesvirus infection [9,32–35] and *Flavobacterium ceti* septicaemia [36].

The Canary Islands are located in a strategically geographic region within the Atlantic Ocean, in which the presence of at least six species from the *Ziphiidae* family has been recorded (https://www.canariasconservacion.org/Zifios-Ziphiidae.htm/ (accessed on 29 June 2021)) [37]. The aim of this study was to realize a retrospective survey on BWs stranded in the Canary Islands in order to determine the presence of CeMV in this subset of the population. Moreover, a phylogenetic analysis was performed in order to analyse the relationships between the obtained sequences with others available in these and other species.

2. Materials and Methods

The availability of the number of animals for this study was possible thanks to the permission for the management of stranded cetaceans granted by the Spanish Ministry of the Environment. Moreover, no animal was sacrificed and no experiments were performed with live animals, so ethical review and approval were waived.

Fifty-five BWs stranded along the coasts of the Canary Islands from November 1999 to May 2017 were included in this study, from which 35 specimens were Cuvier's BWs and 20 specimens belonged to the *Mesoplodon* genus: one True's BW (*Mesoplodon mirus*), two Sowerby's BWs (*Mesoplodon bidens*), seven Blainville's BWs and ten Gervais' BWs (*Mesoplodon europaeus*). A map indicating stranding location of each animal (indicated by its case number and species) was created by the software ArcMap [38] and is shown in Figure 1. The information about each stranding (date, location with coordinates and type) and decomposition stage (grade 1: extremely fresh carcass; grade 2: fresh carcass; grade 3: moderate decomposition; grade 4: advanced decomposition and grade 5: mummified or skeletal remains) [39] is compiled in Table 1, as well as life history data (species, age category, sex and body condition). The nutritional status was classified as good, moderate, poor or emaciated in consonance with the anatomical parameters, such as the observable presence of marked bony process and prominent bones through the skin (the transverse and spinous vertebral processes and ribs), the observable presence of dorso-axial muscular mass and the presence or distribution of fatty tissue in several organs, taking into consideration the species and the age of the animal [22,25]. A complete postmortem examination, following standardized necropsy protocols [39,40], was performed on all of the animals from the study. Collected samples were fixed in 10% neutral buffered formalin solution, embedded in paraffin blocks, sectioned at 5 µm, stained with haematoxylin and eosin (HE) and examined under a light microscope. All the cases included in the present study were diagnosed during routine pathological and cause-of-death analyses of stranded cetaceans at the Division of Histology and Animal Pathology of the Institute for Animal Health (IUSA), Veterinary School, Universidad de Las Palmas de Gran Canaria. Immunohistochemistry was performed in molecular positive samples following a previously described standardized protocol. The tissue sections were incubated with a mouse monoclonal antibody against the nucleoprotein antigen of canine distemper virus (CDV, 1:200 dilution; CDV-NP MAb, VMRD Inc.), for which cross-reactivity with CeMV has been previously reported [41]. Positive controls included laryngeal tonsil from a CeMV-positive striped dolphin stranded in the Canary Islands in 2019. Virological analyses were performed in kept frozen samples (−80 °C).

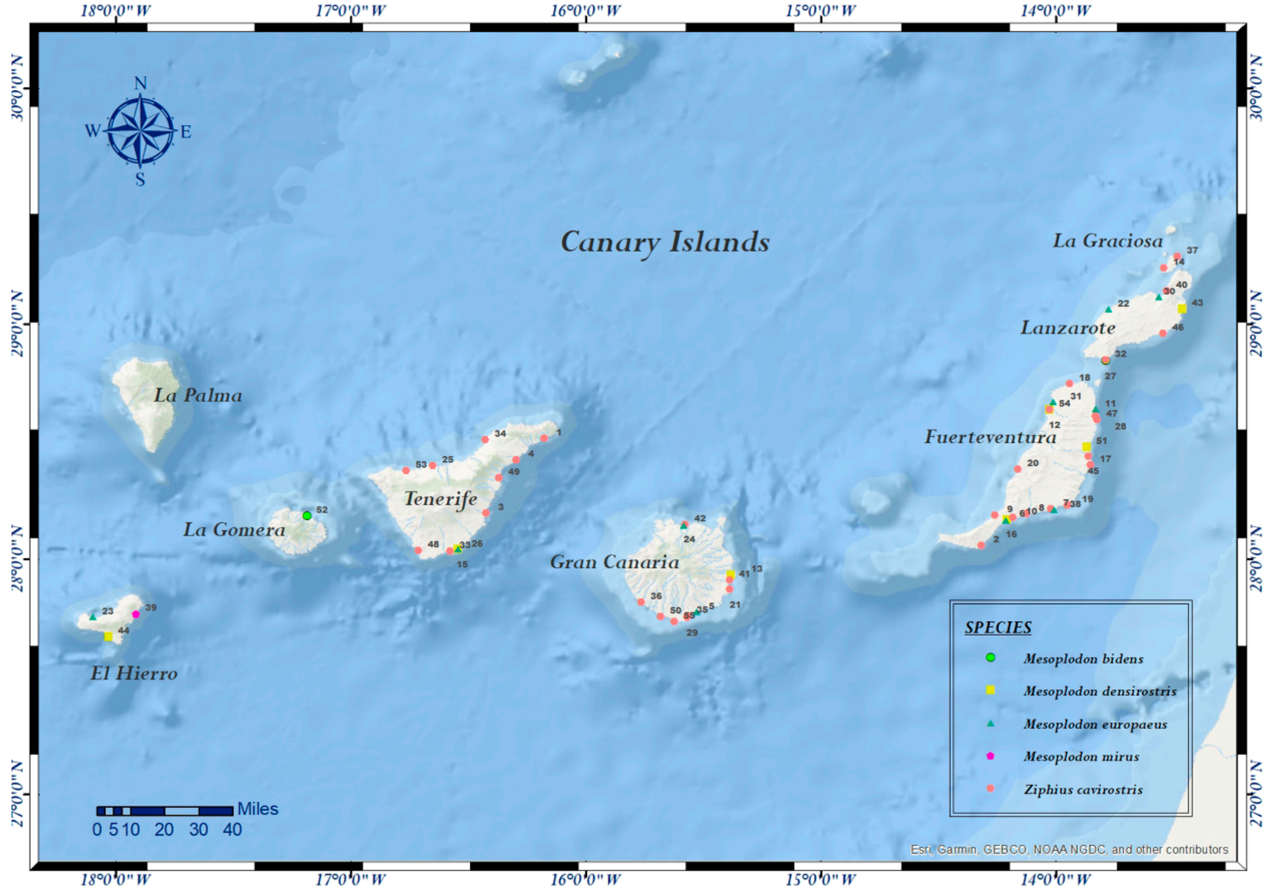

Figure 1. Map of the geographical distribution of beaked whales stranded in the Canary Islands between 1997 to 2017. Stranding sites for each beaked whale is identified by its case number. The species are represented with different colours.

Table 1. Beaked whale specimens included in the present study.

Case N°	ID Code	Species	Sex	Age	SD	SL	Coordinates	SS	DC	NS	Tested Samples	PCR
1	CET 86	*Z. c.*	F	A	27/11/1999	TNF	28.513067382968398, –16.176165295752952	A	3	G	Skin, lung, liver, kidney	C, F
2	CET 103	*Z. c.*	M	J	19/04/2000	FTV	28.056538132454694, –14.317684331100672	D	3	G	Lung, liver, kidney, brain	C, F
3	CET 108	*Z. c.*	F	A	10/06/2000	TNF	28.195070013507404,–16.42181088596981	D	3	G	Skin, skeletal muscle, lung, liver, kidney	C, F
4	CET 113	*Z. c.*	F	S	16/07/2000	TNF	28.420528319456402, –16.295411555275532	D	3	P	Skin, skeletal muscle	C, F, P
5	CET 134	*M. e.*	F	C	28/06/2001	GC	27.77761716736704, –15.52674193489951	A	1	P	Skin, lung, liver, kidney, brain	C, F, P
6	CET 180	*M.d.*	F	A	24/09/2002	FTV	28.167568704666728, –14.206111760665271	A	2	NE	Skin	C, F, P
7	CET 181	*Z. c.*	M	S	24/09/2002	FTV	28.211852124234827, –14.019063839127336	A	2	NE	Skin, skeletal muscle, lung, mediastinal and mesenteric lymph node, liver, kidney, brain, spleen	C, F
8	CET 182	*Z. c.*	M	S	24/09/2002	FTV	28.19146832713967, –14.122994013994195	D	2	NE	Skin, skeletal muscle, lung, liver, mesenteric lymph node, kidney, brain, spleen	C, F, P
9	CET 183	*Z. c.*	M	S	24/09/2002	FTV	28.186564312428384, –14.257397680615142	D	2	NE	Skin, skeletal muscle, lung, liver, mesenteric lymph node, kidney, brain	C, F, P
10	CET 184	*Z. c.*	M	S	24/09/2002	FTV	28.175274186060737, –14.183223454614003	D	2	NE	Skin, skeletal muscle, lung, liver, mediastinal and mesenteric lymph node, kidney, brain, spleen, thyroid	C, F, P
11	CET 185	*M. e.*	F	A	24/09/2002	FTV	28.638713939591398, –13.830348964499422	D	2	NE	Skin, skeletal muscle, lung, liver, mesenteric lymph node, kidney, brain, spleen	C, F, P
12	CET 189	*Z. c.*	F	A	27/09/2002	FTV	28.63498925367787, –14.026753961358423	D	4	NE	Skin, lung, liver, kidney	C, F, P
13	CET 213	*M. d.*	F	A	28/06/2003	GC	27.933119723146724, –15.37973786995963	A	1	P	Skin, skeletal muscle, lung, liver, kidney, brain	C, F
14	CET 236	*Z. c.*	F	C	21/03/2004	LGr	29.23649579711158, –13.538613817871068	D	3	G	Skin, skeletal muscle, lung, liver, kidney, brain	C, F
15	CET 243	*M. d.*	M	A	18/04/2004	TNF	28.040710105511895, –16.5426071324846	A	1	P	Skin, skeletal muscle, lung, liver, kidney	C, F
16	CET 259	*M. e.*	F	J	21/06/2004	FTV	28.164953252754323, –14.212321787452456	D	2	P	Skin, skeletal muscle, lung, liver, kidney, brain	C, F, P
17	CET 264	*Z. c.*	F	ND	23/07/2004	FTV	28.40069477695754, –13.852448567548068	D	4	G	Liver, skeletal muscle, lung, kidney	C, F
18	CET 265	*Z. c.*	M	A	24/07/2004	FTV	28.744473643139884, –13.940991510227075	D	4	G	Skin, skeletal muscle, lung, liver, kidney	C, F
19	CET 294	*Z. c.*	F	A	18/04/2005	FTV	28.228156055155715, –13.949995729432887	D	4	G	Skin, skeletal muscle, lung, liver, spleen	C, F
20	CET 304	*Z. c.*	F	C	13/07/2005	FTV	28.38034460360221, –14.161693075458741	D	2	P	Skin, skeletal muscle, lung, liver, kidney	C, F
21	CET 322	*Z. c.*	M	A	17/02/2006	GC	27.870618611481834, –15.38656422608156	D	4	I	Skin, lung, liver, kidney	C, F
22	CET 333	*M. e.*	F	S	28/03/2006	EH	29.06227107414346, –13.774638588905537	A	2	G	Skin, skeletal muscle, lung, thymus, liver, mesenteric lymph node, kidney, brain, spleen	C, F
23	CET 334	*M. e.*	F	S	28/03/2006	EH	27.755578877787087, –18.09553359589475	A	2	G	Skin, skeletal muscle, lung, liver, kidney, brain, spleen	C, F
24	CET 338	*M. e.*	F	J	06/04/2006	GC	28.14436864892595, –15.581793297520823	D	2	P	Skin, skeletal muscle, lung, liver, blood, mesenteric lymph node, kidney, brain, spleen	C, F
25	CET 352	*Z. c.*	ND	J	06/07/2006	TNF	28.396221323032027,–16.648897697794496	D	3	P	Skin, lung, liver, kidney, brain, spleen	P
26	CET 354	*M. e.*	M	C	28/07/2006	TNF	28.040710105511895, –16.5426071324846	D	4	M	Skin, lung, liver, kidney, spleen	P
27	CET 379	*M. b.*	M	A	16/04/2007	LZ	28.842800242252817, –13.788144917745738	D	2	E	Skin, lung, liver, kidney, brain, spleen	P
28	CET 471	*Z. c.*	F	S	06/11/2008	FTV	28.607731990351333, –13.8297794478201	D	2	G	Lung, kidney, brain, spleen	P
29	CET 503	*Z. c.*	F	A	21/09/2009	GC	27.750297975375215, –15.568322782893084	D	4	I	Skin, lung, kidney, liver	P
30	CET 510	*M. e.*	M	A	14/12/2009	LZ	29.11583432185095, –13.560196813234894	D	2	E	Skin, lung, liver, mesenteric lymph node, kidney, brain	P
31	CET 547	*M. e.*	M	A	29/08/2010	FTV	28.667401632579352, –14.011239799734405	D	4	M	Skin, lung, liver, mesenteric lymph node, kidney, brain	P

Table 1. *Cont.*

Case N°	ID Code	Species	Sex	Age	SD	SL	Coordinates	SS	DC	NS	Tested Samples	PCR
32	CET 576	*Z. c.*	F	A	16/05/2011	LZ	28.84666379719735, –13.788277756973725	D	2	G	Lung, kidney, brain, spleen	F, P
33	CET 579	*Z. c.*	M	S	13/06/2011	TNF	28.031868453191404, –16.575344550886005	D	4	M	Skin, lung, mesenteric lymph node, liver, kidney, brain, spleen	P
34	CET 591	*Z. c.*	F	A	01/11/2011	TNF	28.506326419217828, –16.425276036108105	D	4	I	Skin, lung, prescapular lymph node, liver, kidney, brain, spleen	P
35	CET 593	*Z. c.*	M	A	18/11/2011	GC	27.75629856377708, –15.567180176416969	D	4	G	Skin, lung, prescapular lymph node, liver, kidney	P
36	CET 620	*Z. c.*	M	A	20/05/2012	GC	27.817154801714093, –15.764000859185387	D	4	NE	Skin, lung, liver, kidney, spleen	P
37	CET 624	*Z. c.*	F	A	13/07/2012	LGr	29.286035258319366, –13.481903003501081	D	3	G	Skin, lung, liver, mesenteric lymph node, kidney, brain	F, P
38	CET 631	*M. e.*	M	A	21/10/2012	FTV	28.210771928688324, –14.009197485266776	D	4	G	Skin, lung, penis, palate, esophagus, brain	C, P
39	CET 636	*M. m.*	M	S	30/11/2012	EH	27.765920776532404,–17.910161998928636	D	2	M	Skin, lung, liver, mesenteric lymph node, kidney, brain, spleen	P
40	CET 645	*Z. c.*	M	J	09/02/2013	LZ	29.138139438489326, –13.528173725646699	D	4	M	Skin, liver, brain	F, P
41	CET 680	*Z. c.*	F	N	02/07/2013	GC	27.91040435902637,–15.386896660597422	D	4	M	Skin, lung, liver, intestine, mesenteric lymph node, kidney, brain, spleen	P
42	CET 688	*Z. c.*	F	A	18/11/2013	GC	28.14483414396133, –15.57739880000554	D	4	I	Skin, brain	P
43	CET 695	*M. d.*	F	A	12/07/2014	LZ	29.064020665490503, –13.460099910240649	D	4	M	Skin, lung, liver, mesenteric lymph node, kidney, brain, spleen	P
44	CET 711	*M. d.*	M	S	03/04/2014	EH	27.66937590944239, –18.027604222360484	D	5	P	Skin, lung, liver, mesenteric lymph node, kidney, brain, spleen	P
45	CET 712	*Z. c.*	F	S	28/04/2014	FTV	28.43722859059096, –13.862160820030729	D	4	G	Skin, prescapular lymph node, spleen	P
46	CET 719	*Z. c.*	F	A	06/06/2014	LZ	28.958846751180907, –13.542006915443775	D	3	M	Skin, lung, liver, mesenteric lymph node, kidney, spleen	P
47	CET 720	*Z. c.*	ND	S	10/06/2014	FTV	28.59159662129131,–13.82462012207941	D	4	I	Skin, lung, mesenteric lymph node, liver, kidney, brain	P
48	CET 770	*Z. c.*	M	S	28/07/2015	TNF	28.03511014479708, –16.709505678909764	D	3	G	Skin, lung, liver, intestine, mesenteric lymph node, brain, spleen	P
49	CET 771	*Z. c.*	F	A	05/08/2015	TNF	28.3461443622214, –16.368973513719958	D	2	G	Skin, lung, liver, intestine, mesenteric lymph node, kidney, brain, spleen	P
50	CET 818	*Z. c.*	M	S	16/08/2016	GC	27.756233743172498,–15.67996962889147	D	4	I	Lung, intestine, mesenteric lymph node, kidney, brain, spleen	P
51	CET 824	*M. d.*	F	A	11/11/2016	FTV	28.4768803133933, –13.867047314550653	D	2	E	Skin, prescapular lymph node, liver, kidney, brain, spleen	P
52	CET 827	*M. b.*	F	A	07/12/2016	LG	28.182564817917488, –17.184569356359297	A	4	I	Skin, lung, liver, mesenteric lymph node, intestine, brain, spleen	P
53	CET 833	*Z. c.*	ND	ND	13/02/2017	TNF	28.375804156993976, –16.762765447612193	D	4	I	Skin, lung, liver, mesenteric lymph node, kidney, brain	P
54	CET 852	*M. d.*	F	A	05/05/2017	FTV	28.635507152083715, –14.026812972482025	D	2	P	Skin, lung, liver, mesenteric lymph node, kidney, brain, spleen	P
55	CET 855	*Z. c.*	M	A	22/05/2017	GC	27.734397352882862, –15.622884576151774	D	3	I	Skin, lung, liver, intestine, mesenteric lymph node, kidney, brain, spleen	P

Notes: *Z. c.*: *Ziphius cavirostris; M. d.: Mesoplodon densirostris; M. e.: Mesoplodon europaeus; M. b.: Mesoplodon bidens; M. m.: Mesoplodon mirus*. SD: stranding date; SL: stranding location (GC: Gran Canaria; TNF: Tenerife; FTV: Fuerteventura; LZ: Lanzarote; LG: La Gomera; EH: El Hierro; LGr: La Graciosa); SS: stranding stage; DC: decomposition stage (1 = extremely fresh carcass; 2 = fresh carcass; 3 = moderate decomposition; 4 = advanced decomposition; 5 = mummified or skeletal remains); NS: nutritional status (G: good; M: moderate; P: poor; E: emaciated; NE: not evaluated; I: indeterminate); Sex: F = female; M = male; ND = not determined; age: A = adult; S = subadult; J = juvenile; C = calf, *n* = neonate. PCR: polymerase chain reaction (C: conventional one-step RT PCR [42–44]; F: one step RT-qPCR (F gene) [45]; P: pan RT-qPCR [46].

According to the availability in each case, a total of 319 tissue samples were analyzed: the skin (50/319; 15.67%), lung (49/319; 15.36%), liver (47/319; 14.73%), kidney (46/319; 14.42%), brain (38/319; 11.91%), spleen (29/319; 9.09%), mesenteric lymph node (24/319; 7.52%), skeletal muscle (18/319; 5.64%;), intestine (6/319; 1.88%;), prescapular lymph node (4/319; 1.25%), mediastinal lymph node (2/319; 0.63%), thyroid gland (1/319; 0.31%), thymus (1/319; 0.31%), palate (1/319; 0.31%), oesophagus (1/319; 0.31%), penis (1/319; 0.31%) and blood (1/319; 0.31%). This information is available in Table 1.

Frozen kept samples were thawed and mechanically macerated for a subsequent simultaneous extraction of DNA and RNA by means of a QuickGene R Mini 80 nucleic acid isolation instrument with the DNA Tissue Kit S (QuickGene, Kurabo, Japan) according to the manufacturer's instructions with some modifications: an RNA carrier (Applied Biosystems™, Thermo Fisher Scientific Waltham, MA, USA) was added during the lysis step, as previously published [45].

Molecular detection of CeMV was performed using one or more of three polymerase chain reaction (PCR) methods. (1) was a modified conventional one-step reverse transcription polymerase chain reaction (RT-PCR), which amplifies a fragment of 426 base pairs (bp) from a conserved region of the phosphoprotein (P) gene. Primers and PCR protocol used were the following: ((DMV C: 5′-ATGTTTATGATCACAGCGGT-3′/DMV P2: 5′-ATTGGGTTGCACCACTTGTC-3′) and (94 °C × 4′–45 × (1′ × 94 °C−1′ × 51 °C–1′ × 72 °C)–7′ × 72 °C)). The obtained amplicons were analyzed by means of a 2% agarose gel horizontal electrophoresis [42–44]. (2) was a real-time one-step reverse transcription polymerase chain reaction (RT-qPCR) amplifying a size region of 192-bp from the fusion protein (F) gene using the following primers: (DMVFuF: 5′-GGCACCATAATTAGCCAGGA-3′/DMVFuR: 5′-GCCCAGATTTGTGCCTACAT-3′) and the PCR protocol (30′ × 48 °C–95 °C × 3′–40 × (95 °C × 3″–60°C × 30″)) [45] and (3) was a PAN RT-qPCR method based on SYBRN® green dye [46] that successfully detects GDMV, PWMV and DMV strains. The primer set (Forward PAN-F (5′-CCTCTAACAGGGGATCT(A/G)CTC-′3) and Reverse PAN-R (5′-CCTGTGCCCTTTTTAATGGA-′3)) amplifies 205 bp from a region of the phosphoprotein (P) gene. The PCR protocol used was as follows: 50 °C × 10′–95 °C × 1′–40 × (95 °C × 10″–60 °C × 30″). The information about which PCR method was used for the detection of CeMV in each animal is specified in Table 1. A negative control (non-template) and an amplification-positive control (known cetacean morbillivirus RNA previously obtained in our laboratory) were added in each ADN/ARN extraction and PCR protocols described above. Genomic DNA digestion was performed in the total RNA/DNA extractions followed by a second purification following the same protocol as previously described. RNA extractions were then subject to a one-step RT-qPCR that amplifies the housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as previously described [47], in order to ensure the high quality of the RNA [48]. In addition, all the samples were checked for the presence of herpesvirus by means of a pan-herpesvirus conventional nested PCR based on the DNA polymerase gene [35,49].

Previous to the Sanger sequencing method, the PCR products were purified using Real Clean spin kit (REAL®, Durviz, S. L., Valencia, Spain). BLAST algorithm (www.ncbi.nlm.nih.gov/blast/Blast.cgi/ (accessed on 10 November 2021)) [50] was used to compare the obtained amplicons with other somewhat similar sequences published in GenBank. The sequences were aligned using ClustalW algorithm through software MEGA X [51]. A total of 102 CeMV nucleotide sequences based on the P gene were recovered from GenBank to construct the phylogenetic tree, where three canine distemper virus (CDV) sequences were used to root the phylogram as an outgroup. The phylogenetic tree was constructed using the Maximum Likelihood Method and the Kimura 2-parameter model with a discrete Gamma distribution to model the evolutionary rate differences among sites (5 categories (+G, parameter = 0.7797)). The Bootstrap method (500 replicates) was applied to assess the reliability of the tree.

A phylogenetic tree based on the F gene was also created, which was constructed using the Maximum Likelihood Method and the Tamura 3-parameter model with a discrete

Gamma distribution to model the evolutionary rate differences among sites (5 categories (+G, parameter = 0.5319)). A Bootstrap test from 500 replicates was also implemented. This analysis involved 52 nucleotide sequences, where two Peste des petits ruminants virus (PPRV) and two Phocine distemper virus (PDV) sequences established the root of the tree.

The DMV sequences from P gen and F gen were identified from this study and were deposited in GenBank under accession nos. OM055653 and OM055654, respectively.

3. Results

The presence of CeMV was detected in one animal (1/55; 1.82%), a subadult male Cuvier's BW stranded to death in Fuerteventura in September 2002 in a good state of preservation (code 2, CET 182). Positivity was achieved in the skin sample (1/319; 0.3%). All the tested tissue samples from this animal were negative for the herpesvirus infection [35].

This Cuvier's BW was one of the 14 BWs (designed as BW-3) stranded on the beaches of Fuerteventura and Lanzarote islands on 24 September 2002, temporally associated with naval exercises involving acoustic (sonar) activities (manoeuvers called Neo-Tapon 2002) [19]. Macroscopically, no systemic, inflammatory or neoplastic processes were noted, although the carcass showed severe diffuse congestion and haemorrhages, especially around the acoustic jaw fat, ears, brain and kidneys. Gas bubble–associated lesions and fat embolism were observed in the vessels and parenchyma of vital organs in the histopathological study (DCS) [19]. No pathogens were identified in routine aerobic bacterial cultures of the brain, lungs and spleens from this specimen [19].

The housekeeping gene was not amplified in samples from 11 animals of the 55 analyzed in the present study (20%). The RNA from these negative samples was too degraded for causes determining causes due to descomposition stages or the time elapsed since it was taken. Thus, only 44 animals were considered validated for the CeMV molecular analyses.

3.1. Nucleotide Identity

Skin samples tested positive for CeMV by means of two real time PCRs: a PAN RT-qPCR (P gene), as indicated by means of a visualization of an amplification curve at the 33rd cycle and a post-amplification melting-curve of 81–82 °C and a RT-qPCR (F gene), visible at the 31st amplification cycle and a post-amplification melting curve of 80 °C. A further sequencing of the P gene fragment (164 bp excluding primers) revealed that it had a 100% similarity (100% query cover) to DMV detected in two striped dolphins: one stranded in 2007 on the north-eastern coast of Portugal (GenBank acc. no. KP835995) and another stranded in the Central-East Atlantic Ocean (Canary Islands) in 2002 (GenBank acc. no. KJ139451).

3.2. Phylogenetic Analyses

The nucleotide phylogenetic analysis based on the P gen showed that the obtained tree presents five main branches, where DMV, BWMV, PWMV, PMV and GDMV strains are clearly clustered in their respective clades (Figure 2). The DMV clade is supported by a Bootstrap value of 75 and contains a polyphyletic group of 71 sequences detected in 12 different species of odontocetes and mysticetes from the Mediterranean Sea (n = 39), the North-East Atlantic Ocean (n = 11), the Central-East Atlantic Ocean (n = 8), the North Sea (n = 7), the West Atlantic Ocean (n = 4) and the Pacific Ocean (n = 2) from 1990 until 2017.

Specifically, the first documented DMV sequences date from 1990 (n = 7), and since then, there are no more available sequences until 2002 (n = 1). From 2005 to 2017, there is at least one available sequence every year. Furthermore, from the phylogram analysis, we can observe two clearly separate clades within the DMV strain: one containing the sequences detected in the white-beaked dolphin species in the North Sea in 2007 and 2011 and in a striped dolphin stranded in Italy in 2016 and another larger clade containing two subclades.

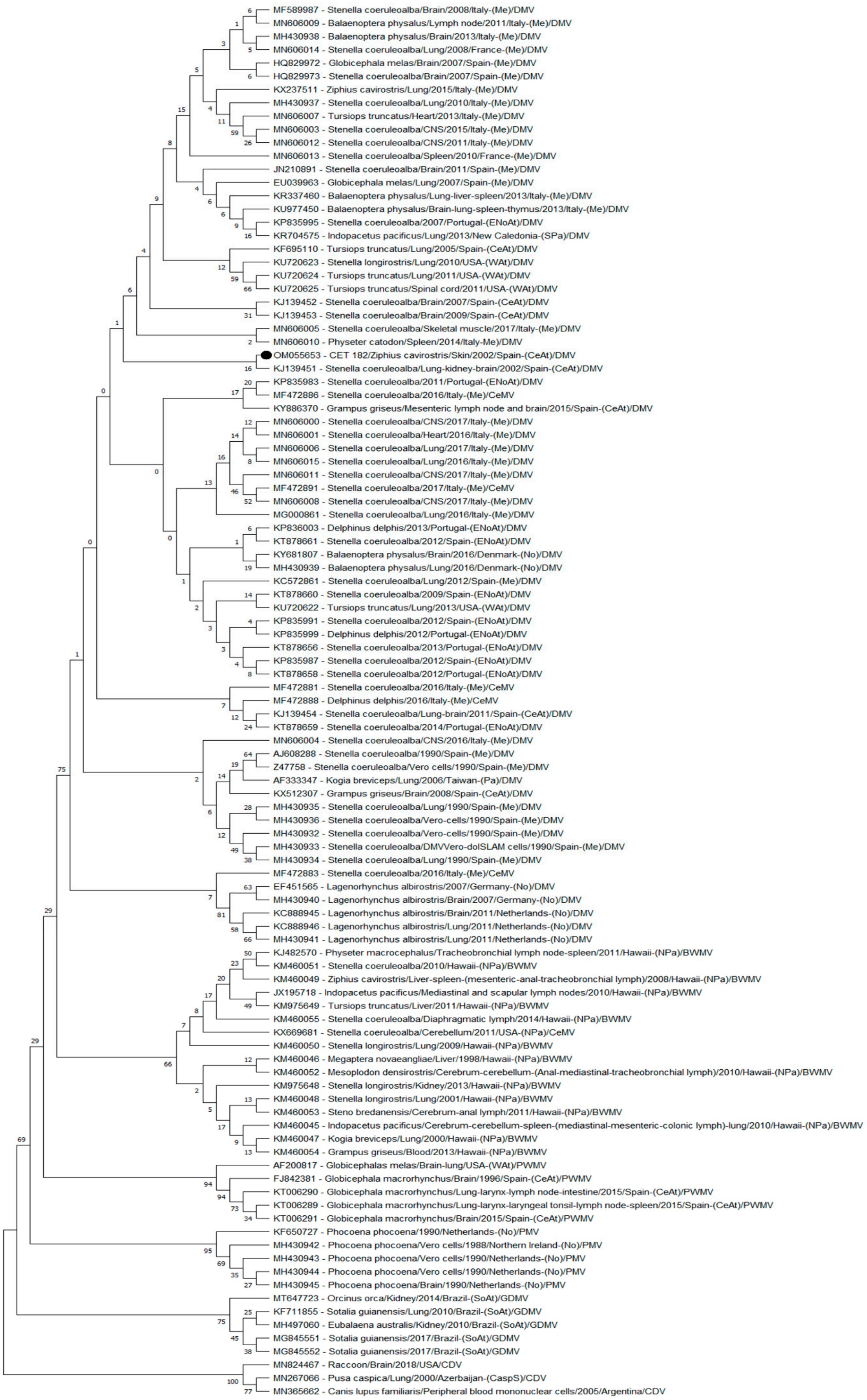

Figure 2. Maximum likelihood phylogenetic tree for the nucleotide sequences of the phosphoprotein (P) gene. The phylogenetic tree consists of 102 sequences from reported cases of cetacean morbillivirus.

To construct the tree, were designed the Neighbour-Join and BioNJ algorithms along with the Kimura 2-parameter model and Gamma distribution to model the evolutionary rate differences among sites [5 categories (+G, parameter = 0.7797)]. The Bootstrap method was performed to resample 500 replicates and evaluate the reliability of the tree. The accession number from GenBank, the host, the sample of detection, the date of collection, and the geographic area of stranding were used to identify the nucleotide sequences. Abbreviations: ENoAt (Northeast Atlantic Ocean); WAt (West Atlantic Ocean); CeAt (Central Atlantic Ocean); SoAt (South Atlantic Ocean); Me (Mediterranean Sea); Pa (Pacific Ocean); NPa (North Pacific Ocean); No (North Sea); CaspS (Caspian Sea).

In one of these, there are the sequences detected in striped dolphins during the first CeMV epizootic event in the Mediterranean Sea in the early 90s plus three sequences detected in a striped dolphin stranded in Italy in 2016 in a pygmy sperm whale (*Kogia breviceps*) stranded in the Pacific in 2006 and in a Risso's dolphin (*Grampus griseus*) stranded in the Central-East Atlantic Ocean in 2008, respectively. In the other one, there are the rest of the DMV sequences detected until a date that includes the only two previous DMV sequences from BWs. The two DMV sequences previously detected in BWs are located in separate branches within the phylogram; the sequence detected in a Cuvier's BW stranded in the Mediterranean Sea (Italy) in 2015 (GenBank acc. no. KX237511) is clustered with four sequences detected in three striped dolphins and one bottlenose dolphin (*Tursiops truncatus*) stranded on the coast of Italy in a period of five years (2010, 2011, 2013 and 2015, GenBank acc. nos. MH430937, MN606007, MN606003 and MN606012), and the sequence detected in a Longman's BW in New Caledonia in 2013 is clustered with the sequence previously detected in a striped dolphin stranded in 2007 in the North-East Atlantic Ocean (Portugal, GenBank acc. no. KP835995), which is being shown to be 100% similar in a BLAST search with the sequence identified in our study. The isolated sequence obtained from this study was clustered with a single sequence, which was detected in a striped dolphin stranded in the Central-East Atlantic Ocean (Canary Islands) in 2002 (GenBank acc. no. KJ139451).

The obtained F gene fragment (125 bp excluding primers) had a 100% percentage of similarity (100% query cover) with 14 cetacean sequences (GenBank acc. nos. MT066174, MH430936, MH430935, MH430934, MH430933, MF589987, KU720625, KU720624, KU720623, MN606014, HQ829972, AJ608288, AJ224704 and Z30086). Most of these sequences (11/14; 78.6%) were detected in the Mediterranean Sea in the early 90s (8/14; 57.1%) and in the second Mediterranean epizootic event (3/14; 21.4%). The other three sequences (21.4%) were detected on the West Atlantic coast (USA) in 2010 and 2011.

The phylogenetic nucleotide sequence analysis based on the F gene (Figure 3) showed a DMV clade supported by a Bootstrap value of 81 conformed by a polyphyletic group of 42 sequences detected in seven cetacean species and one harbour seal (*Phoca vitulina*). No other F gene sequences obtained from BWs are available in GenBank. The obtained BW sequence from this study was exclusively clustered with a sequence detected in a Risso's dolphin (*Grampus griseus*) stranded in the Central-East Atlantic Ocean (Canary Islands) in 2008 (GenBank acc. no. KX512308).

3.3. Histopathology, Immunohistochemistry

To date, only frozen skin tissue samples from this case remained available for the histopathological study. At the microscopic level, neither CeMV-associated lesions nor immunostainings for CDV were observed, although ballooning, interpreted as a freeze artefact, was present in most of the keratinocytes of the stratum spinosum (Figure 4A,B). The immunolabelling of CDV was observed in the epithelial cells from the positive control (laryngeal tonsil sample from a CeMV-positive striped dolphin stranded in the Canary Islands in 2019, Figure 4C).

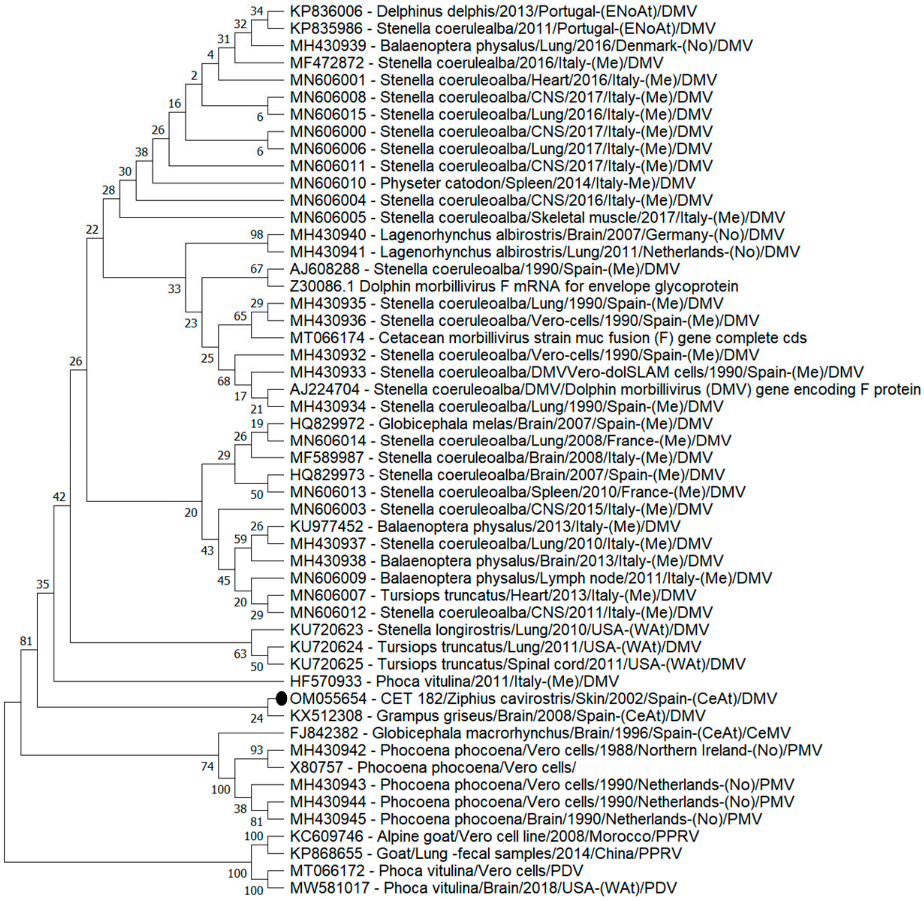

Figure 3. Maximum likelihood phylogenetic tree for the nucleotide sequences of the fusion protein (F) gene. The phylogenetic tree consisting of 52 sequences from reported cases of cetacean morbillivirus. To construct the tree, we designed the Neighbour-Join and BioNJ algorithms along with the Tamura 3-parameter model and Gamma distribution to model the evolutionary rate differences among sites (five categories (+G, parameter = 0.5319)). The Bootstrap method was performed to resample 500 replicates and evaluate the reliability of the tree. The accession number from GenBank, the host, the sample of detection, the date of collection and the geographic area of each stranding were used to identify the nucleotide sequences. Abbreviations: ENoAt (North-East Atlantic Ocean); WAt (West Atlantic Ocean); CeAt (Central Atlantic Ocean); Me (Mediterranean Sea); No (North Sea).

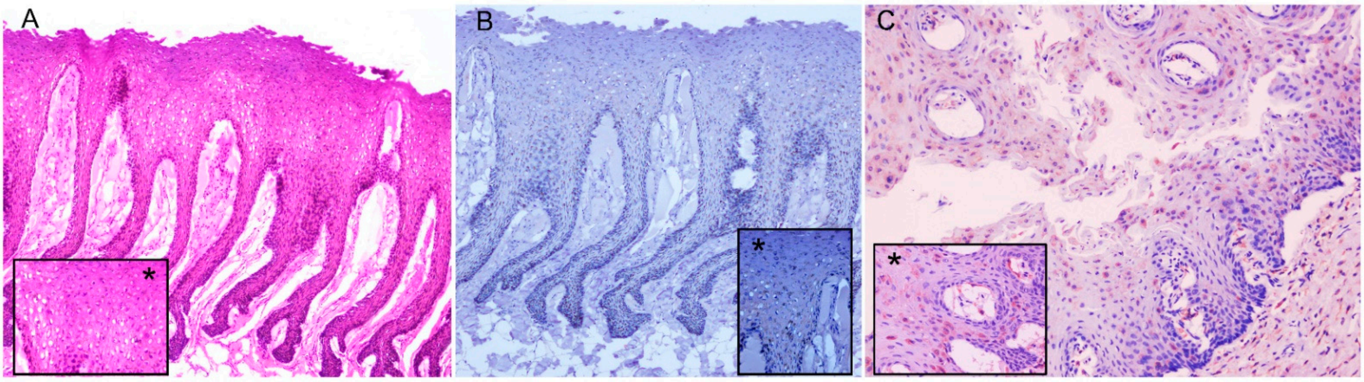

Figure 4. Histological and Immunohistochemistry evaluation of PCR-positive skin sample from a Cuvier's Beaked whale (CET 182). (**A**) Histopathology. Most of the keratinocytes of the stratum spinosum exhibit ballooning (freeze artefact). HE stain, ×10. Inset: Detail of the ballooning (*). HE

stain, ×40. (**B**) Immunohistochemistry. CDV immunostaining was not observed. Immunohistochemistry stain, X 10. Inset: detail of lack of specific immunostaining against CDV in keratinocytes (*). Immunohistochemistry stain, ×40. (**C**) Immunohistochemistry. Positive control for CDV antibody in laryngeal tonsil sample. Immunostaining was observed in the cytoplasm of epithelial cells. Immunohistochemistry for morbillivirus using an antibody to CDV nucleoprotein., ×20. Inset: detail of specific immunostaining against CDV in epithelial cells (*). ×60.

4. Discussion

CeMV was detected in one of the 55 BWs analyzed for the presence of this pathogen in the Canary Islands. Specifically, the virus (DMV strain) was present in the skin sample of a subadult male Cuvier's BW, representing the second molecular confirmation of a DMV infection in this species. The detection of DMV in a Cuvier's BW was first reported in Italy in the lung sample of a male calf stranded in 2015 [15], 13 years after the sample from our study was collected. Therefore, the retrospective analysis of archived tissue samples carried out in our study exposed that this was the earliest confirmed occurrence of DMV in the Cuvier's BW species.

Positivity was achieved in the skin sample by means of two real-time PCRs but not with the conventional one. The skin sample had high cycle-threshold values in both qPCRs and therefore low viral loads, which could be only detected with test methods with higher sensitivities when compared to more traditional assays [52]. CeMV has been previously detected in the skin samples of marine mammals with and without associated lesions [41,53,54]. No lesions were observed in the skin sample of the Cuvier's BW from our study. In cetaceans, the effects of CeMV are widely variable from causing epidemics to subclinical infections. According to previous studies about CeMV in BWs, even if a study does not describe histopathological findings in detail, it seems that the infection does not cause severe pathological effects in these species [13–15]. The only exception is the first published case of a BWMV infection in a Longman's BW stranded in Hawaii in 2010, which presented an associated nonsuppurative encephalitis but which was also co-infected with the herpesvirus [9]. In addition to the herpesvirus, secondary infections or co-infections with other viruses, bacteria, fungi, protozoans or parasites have been frequently described [2,48,55–59]. In our study, the presence of bacterial pathogens and a herpesvirus infection was ruled out [19,35].

The level of detection of CeMV in stranded BWs in the Canary Islands (1.82%; 1/55) in an 18-year period (1999–2017) is low compared with previous studies in other geographical regions, which range from 5.7% to 48.1% (Table 2). This variability in the level of detection is partially due to the fact that some of those retrospective studies included eligibility (inclusion) criteria or morbillivirus-related lesions, species classically considered DMV hosts, such as striped and bottlenose dolphins and/or areas and years in which some epizootic events or outbreaks were involved [1,48,60]. However, the absence of DMV detection in other samples from our study does not rule out the presence of DMV in such samples. The assessment of RNA integrity is a critical first step for detecting false-negative samples. Current epidemiological knowledge of CeMV in Canarian waters indicates the presence of some inter-epizootic sporadic endemic presentations of the infection with the detection of the virus in 1996, 2002, 2005, 2007–2009, 2011–2012, 2015 [44,59,61,62], 2016 and 2018–2020 (unpublished data). The exception is an unusual mortality event in 2015 involving short-finned pilot whales and the PWMV strain [63].

Table 2. Summary of the percentages of cetacean morbillivirus prevalences reported in other species, geographical zones and dates.

Region	Period	Species	Diagnostic Test	% Prevalence	References
Portuguese and Galician coasts (North-East Atlantic Ocean)	2004–2015	*Stenella coeruleoalba*, *Delphinus delphis*	PCR	5.7% (16/279)	[64]
Southeastern coast of USA (North-West Atlantic Ocean)	2003–2007	*Tursiops truncatus*	Serology	9.8% (12/122)	[65]
The Canary Islands (Central East Atlantic Ocean)	2003–2015	*Grampus griseus*	PCR	16.6% (2/12)	[66]
Northern Gulf of Mexico and USA (West Atlantic Ocean)	2010–2014	*Tursiops truncatus*	PCR Serology Histopathology + PCR	9.9% (14/142) stranded cetaceans; 1% (1/83) free-ranging live 29% (5/7) of live stranded 23% (23/102) of live free-ranging 6.6% (9/136)	[67]
Italy (Mediterranean Sea)	2006–2014	*Balaenoptera physalus*	PCR Serology IHC	21.74% (5/23)	[68]
Hawaii (Pacific Ocean)	1997–2014	*Megaptera novaeangliae*, *Kogia breviceps*, *Stenella longirostris*, *Ziphius cavirostris*, *Stenella frontalis*, *Indopacetus pacificus*, *Stenella coeruleoalba*, *Mesoplodon densirostris*, *Tursiops truncatus*, *Physeter macrocephalus*, *Steno bredanensis*, *Grampus griseus*	PCR	24% (15/62)	[13]
Italy (Mediterranean Sea)	1998–2014	*Stenella coeruleoalba*, *Tursiops truncatus*, *Balaenoptera physalus* and *Globicephala melas*	Serology	32.8% (23/70)	[69]
Central California coast (Pacific Ocean)	2000–2015	*Phocoena phocoena*, *Delphinus capensis*, *Lagenorhynchus obliquidens*, *Stenella coeruleoalba*, *Grampus griseus*	PCR (1/11) Serology (3/11)	36.36% (4/11)	[70]
Brazil (South Atlantic Ocean)	2010–2017	*Eubalaena australis*	PCR	37.5% (3/8)	[71]
Australia (Pacific Ocean)	2005–2011	*Peponocephala electra*, *Tursiops aduncus*, *Lagenodelphis hosei*, *Tursiops truncatus*, *Balaenoptera edeni*	Serology	48.1% (13/27)	[72]

The isolate from this study is one of the only two earliest CeMV sequences available in GenBank detected after the first epizootic event on the Spanish Mediterranean coast in 1990–1992 [73], both of them detected in the same year in the Canary Islands in two different cetacean species and highly similar. The individual from our study stranded in 2002, and the obtained sequences showed the closest relationship with another DMV detected in cetaceans from the Canary Islands (with a striped dolphin stranded in the same year with a systemic chronic infection (GenBank acc. no. KJ139451) [61] according to the nucleotide sequence alignment and phylogenetic analysis of the P gene) and with a Risso's dolphin stranded in 2008 (with chronic localized CeMV encephalitis (GenBank acc. no. KX512398) [66] according to the phylogenetic analysis of the F gene). This information seems to support a previous hypothesis of a cross-species infection [15,44,61,66,67,69,74,75]. The P gene nucleotide analysis also showed that the isolate is highly similar to one detected in a striped dolphin stranded on the North-East coast of Portugal in 2007 (GenBank acc. no. KP835995) [64] in the middle of the second epizootic event on the Spanish Mediterranean coast [55,76,77], which also clustered with samples from the Mediterranean Sea from 2007 to 2013. The F gene nucleotide analysis showed that very similar sequences were detected in the Mediterranean Sea in the first and second Mediterranean epizootic events. Taken together, these results are consistent with the previous supported idea that CeMV is endemic in the Central and North-East Atlantic and that interanimal transmissions might occur through the Strait of Gibraltar. This viral exchange could cause Mediterranean outbreaks and unusual mortality events in that region due to the relatively high density of animals, specifically striped dolphins, and their gregarious behaviour [57,61,64,76,78–85]. In addition, the P gene sequence from Portugal (GenBank acc. no. KP835995) is clustered with the DMV isolate detected in a Longman's BW from New Caledonia (GenBank acc. no. KR704575) as previous studies have indicated [64]. Thus, both DMV sequences detected in BWs from the Central East Atlantic in 2002 and the South-West Pacific in 2013, respectively, show high similarities to the sequence detected in a striped dolphin from the coast of Portugal in 2007, which is also similar to sequences from the Mediterranean from 2007 to 2013. It has been previously reported that undefined cetacean species, due to their extensive patterns of migration and their interactions with certain dolphin populations, may mediate the trans-oceanic spread of CeMV, driving the dispersal and evolution of this virus [72,75,86]. The Cuvier's BW species has a worldwide distribution, featured in almost all temperate, sub-tropical, tropical, sub-polar and polar waters [87]. Moreover, this species presents migrating behaviours in the Atlantic Ocean, and it could probably act as a reservoir spreading the disease into more susceptible populations as previously proposed for fin whales [75,88,89].

5. Conclusions

We present the first molecular confirmation of a DMV infection in a Cuvier's BW, although new retrospective studies worldwide could add further evidence of prior cases of infection in this species. The isolate dates from 2002, 10 years after the first Mediterranean epizootic event took place. This is also the second DMV sequence available since then, both of them detected the same year (2002) and collected from stranded cetaceans of different species in the Central East Atlantic Ocean (Canary Islands). These two sequences were identical, indicating that the cross-species infection is not as new as previously suggested.

The epidemic status of the archipelago is that of an endemic situation of several cetacean species rather than an epidemic, although the circulation of strains similar to those later detected in the North-East Atlantic, the Mediterranean and the South-West Pacific indicate that the migration pattern of some of these species could disseminate the virus to other areas and cause epizootic or unusual mortality events.

Author Contributions: Conceptualization, E.S. and A.F.; methodology and formal analysis, I.F.-J., E.S., A.F., M.A., S.S.-G., A.C.-R., C.M.S.-S. and J.D.L.F.; writing—original draft preparation, I.F.-J. and E.S.; review and editing, E.S. and A.F.; supervision, E.S., A.F. and M.A.; funding acquisition, A.F., E.S. and M.A. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The availability of the number of animals for this study was possible thanks to the permission for the management of stranded cetaceans granted by the Spanish Ministry of the Environment (SGPM/BDM/AUTSPP/70/2019). Moreover, neither animal was sacrificed and not experiments were performed with live animals, so ethical review and approval were waived.

Informed Consent Statement: Not applicable.

Data Availability Statement: The DMV obtained sequences from a Cuvier's beaked whale have been deposited in GenBank (under accession numbers: OM055653, OM055654).

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Conflicts of Interest: The authors declare no conflict of interest.

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5.3. ARTÍCULO 3:

Cerebral nasitremiasis in a Blainville's beaked whale (*Mesoplodon densirostris*) stranded in the Canary Islands.

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Cerebral nasitremiasis in a Blainville's beaked whale (*Mesoplodon densirostris*) stranded in the Canary Islands

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ABSTRACT

Ten species within the genus *Nasitrema* (subfamily *Nasitrematinae,* family *Brachycladiidae*) have been reported infecting a wide variety of odontocetes worldwide, although there is still a lack of information about their presence in beaked whales (BWs). *Nasitrema* spp. are commonly described inhabiting the pterygoid sinus, the tympanic cavities, and the middle and inner ear; although aberrant migrations through the brain have been also reported. This trematode may cause different type of lesions, ranging from mild to severe saculitis, neuritis, otitis, and/or meningoencephalitis that may impede cetaceans to survive in the wild, resulting in incoordination, loss of equilibrium, and echolocation dysfunction ending in a stranding event. The presence of *Nasitrema* sp. was found in an adult female Blainville beaked whale stranded death in Fuerteventura, Canary Islands, on November 2016. The most relevant gross finding was a severe chronic-active multifocal pyogranulomatous and necrotizing encephalitis. Histologically, multiple areas of necrosis, pyogranulomatous and eosinophilic inflammation, haemorrhages and occasional cholesterol crystals were found associated with parasitic structures compatible with an adult trematode and its eggs. Molecular analysis, based on a quantitative polymerase chain reaction (qPCR) of the brain tissue sample detected 99% homology with a partial sequence of the NADH dehydrogenase subunit 3 (ND3) gene of *Nasitrema delphini*. In addition, liver, kidney, prescapular lymph node and brain samples were positive to herpesvirus (conventional nested PCR). Evidence of the presence of this parasite was not found in any of the 54 beaked whales ($n = 54$) stranded on the Canary Islands between 1999 and 2017, specifically 35 Cuvier's BWs and 19 specimens belonging to the *Mesoplodon* genus. To our knowledge, the current study represents the first description of a nasitremiasis in a member of the *Ziphiidae* family.

1. Introduction

Digeneans of the family *Brachycladiidae* (Phylum Platyhelminthes, class Trematoda) are restricted to marine mammals, from which ten species within the genus *Nasitrema*, (subfamily *Nasitrematinae*) (WoRMS Editorial Board, 2022) have been reported infecting a wide variety of toothed whales (odontocetes) worldwide (Table 1). *Nasitrema* spp. are commonly described inhabiting the pterygoid sinus, the tympanic cavities (Arbelo et al., 2013; Cowan et al., 1986; Dailey and Walker, 1978; Degollada et al., 2002; Díaz-Delgado et al., 2018; Lim et al., 2016; Neiland et al., 1970; Oliveira et al., 2011; Schwab, 1985; Shiozaki and Amano, 2017; Walker and Cowan, 1981) and the middle and inner ear (Dailey and Ridgway, 1976; Degollada et al., 2002; Díaz-Delgado et al., 2018), although ova and occasionally adults of these parasites have also been found damaging the vestibulocochlear nerve (VIII cranial nerve) (Degollada et al., 2002; Morimitsu et al., 1992; Morimitsu et al., 1987; Morimitsu et al., 1986; Oliveira et al., 2011) and the brain (Cowan et al., 1986; Dailey, 1985; Dailey and Walker, 1978; Degollada et al., 2002; Díaz-Delgado et al., 2018; Lewis and Berry, 1988; O'Shea et al., 1991; Phillips and Suepaul, 2017; Ridgway and Murray, 1972; Sierra et al., 2020). In those cases, *Nasitrema* may cause different type of lesions (Arbelo et al., 2013; Díaz-Delgado et al., 2018; Morimitsu et al., 1992; Morimitsu et al., 1987; Morimitsu et al., 1986; Ridgway and Murray, 1972), ranging from mild to severe saculitis, neuritis, otitis, and/or meningoencephalitis (St. Leger et al., 2018) that may impede cetaceans to survive in the wild, resulting in incoordination, loss of equilibrium,

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Table 1
Summary of reported worldwide cetacean species with nasitremiasis.

Cetacean species	References
Finless porpoise (*Neophocaena phocaenoides*)	(Ozaki, 1935; Yamaguti, 1951)
Dall's porpoise (*Phocoenoides dalli*)	(Cowan et al., 1986; Dailey and Walker, 1978; Yamaguti, 1951)
Short-finned pilot whale (*Globicephala macrorhynchus*)	(Degollada et al., 2002; Morimitsu et al., 1987; Neiland et al., 1970; Yamaguti, 1951)
False killer whales (*Pseudorca crassidens*)	(Morimitsu et al., 1987; Neiland et al., 1970)
Pacific white-side dolphin (*Lagenorhynchus obliquidens*)	(Dailey and Walker, 1978; Kikuchi et al., 1987; Lewis and Berry, 1988; Neiland et al., 1970; Ridgway and Murray, 1972)
Northern right whale dolphin (*Lissodelphis borealis*)	(Cowan et al., 1986; Dailey and Walker, 1978; Neiland et al., 1970)
Common dolphin (*Delphinus delphis*)	(Cowan et al., 1986; Dailey and Walker, 1978; Degollada et al., 2002; Díaz-Delgado et al., 2018; Fraija-Fernández et al., 2016; Neiland et al., 1970; Ridgway and Murray, 1972; Walker and Cowan, 1981)
Atlantic bottlenose dolphin (*Tursiops truncatus*)	(Arbelo et al., 2013; Dailey and Ridgway, 1976; Degollada et al., 2002; Díaz-Delgado et al., 2018; Kumar et al., 1975; Ridgway and Murray, 1972; Sierra et al., 2020)
Striped dolphin (*Stenella coeruleoalba*)	(Dailey and Walker, 1978; Degollada et al., 2002; O'Shea et al., 1991; Oliveira et al., 2011)
Pygmy killer whale (*Feresa attenuata*)	(Fraija-Fernández et al., 2015; Schwab, 1985)
Melon-headed whale (*Peponocephala electra*)	(Morimitsu et al., 1986; Phillips and Suepaul, 2017)
Pantropical spotted dolphin (*Stenella attenuata*)	(Forrester, 1991; Oliveira et al., 2011)
Rough-toothed dolphin (*Steno bredanensis*)	(Arbelo et al., 2013; Degollada et al., 2002; Ebert and Valente, 2013; Forrester, 1991)
Risso's dolphin (*Grampus griseus*)	(Morimitsu et al., 1992)
Pygmy sperm whale (*Kogia breviceps*)	(Degollada et al., 2002)
Atlantic spotted dolphin (*Stenella frontalis*)	(Degollada et al., 2002)
Spinner dolphin (*Stenella longirostris*)	(De La Fuente Marquez et al., 2016)
Guiana dolphin (*Sotalia guianensis*)	(Ebert and Valente, 2013)
Long-beaked Common Dolphin (*Delphius capensis*)	(Lim et al., 2016)
Long-finned Pilot Whale (*Globicephala melas*)	(Fraija-Fernández et al., 2016)
Narrow-ridged finless porpoise (*Neophocaena asiaeorientalis*)	(Shiozaki and Amano, 2017)

and echolocation dysfunction ending in a stranding event (Dailey and Walker, 1978; Lewis and Berry, 1988; Morimitsu et al., 1992; Morimitsu et al., 1987; Morimitsu et al., 1986; O'Shea et al., 1991).

Up to date, the life cycle of this trematode remains a subject of discussion, although it has been recently reported that *Brachycladiidae* are closely related to *Acanthocolpidae*, parasites of marine teleost fishes that typically possess three-host life cycles with gastropods acting as the first intermediate hosts and either fishes or bivalves acting as the second intermediate hosts (Kremnev et al., 2020). The presence of adults and eggs of *Nasitrema attenuata* in faeces reinforces the hypothesis that infection could be acquired through consumption of infected fish containing the larval stages of the parasite (Kleinertz et al., 2014; St. Leger et al., 2018).

The *Ziphiidade* family is widely distributed throughout the planet (MacLeod, 2018) and consists of 22 species and 6 genera, although a new beaked whale (BW) species, *Mesoplodon eueu*, has been recently described based on its genetic and morphological features (Carroll et al., 2021). The Canary Islands constitute an important geographical enclave located in the Atlantic Ocean, with an enormous diversity of marine mammals (Canarias Conservación, 2018a). Six different species of BWs, including hold species with a presence throughout the year and/or transient populations, have been recorded in this archipelago (Canarias Conservación, 2018b). Beaked whales are considered exceptional long and deep divers and because of their pelagic habits and by their scarcity, among other reasons, parasitological data from the *Ziphiidae* family are scarce (Di Azevedo et al., 2016; Díaz-Delgado et al., 2016; Fraija-Fernández et al., 2018; Jerdy et al., 2022; Paggi et al., 1998). Descriptions of the presence of trematodes of the family *Brachycladiidae* in these cetacean species are limited to liver parasitization, with no previous reports of Nasitremiasis (Fraija-Fernández et al., 2014; Nakagun et al., 2018; Nakagun and Kobayashi, 2020).

The present research represents the first description of cerebral nasitremiasis in a BW, which is associated with pyogranulomatous and necrotizing encephalitis severe enough to cause the stranding and/or death of the animal. Based on this finding, and due to the high number of beaked whale individuals analysed by our group, we reviewed in more detail all the specimens of the *Ziphiidae* family of our database for the occurrence of the presence of eggs, larvae and/or adults of *Nasitrema* spp.

2. Material and methods

A 454 cm-length, 814 kg-weight, adult female of Blainville's beaked whale (*Mesoplodon densirostris*) (CET 824), was found stranded dead at Playa Blanca, Fuerteventura, Canary Islands (N28° 28′ 33.665” N, 13° 52′ 0.033” W) on the afternoon of November 11th, 2016. The carcass was in a fresh conservation status (code 2) following the subsequent categories of decomposition stage: grade 1: very fresh; grade 2: fresh; grade 3: moderate autolysis; grade 4: advanced autolysis and grade 5: very advanced autolysis (Arbelo et al., 2013; Kuiken and García-Hartmann, 1991). The animal was transported, by the environmental agents of the Cabildo of Fuerterventura, to the Rescue Centre of La Oliva, Fuerteventura, and preserved at 4 °C until the next morning. The necropsy was performed by the veterinary staff of the Institute of Animal Health (IUSA) of the Universidad de Las Palmas de Gran Canaria following a complete standardized necropsy protocol for stranded cetaceans (Kuiken and García-Hartmann, 1991). The post-mortem study was possible thanks to the permission for the management of stranded cetaceans conceded by the Spanish Ministry of the Environment (SGPM/BDM/AUTSPP/70/2019). Physical development was estimated according to the total length of the animal (Perrin et al., 2009) and the histological study of the gonads (Geraci et al., 2005). Representative tissue samples from all the organs were taken and immersed in 10% neutral buffered formalin, carved, routinely processed, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (HE) for light microscopic examination (Olympus BX51, Tokyo, Japan) which use a Camara software for DP21 (Olympus DP21, Tokyo, Japan). Required additional stains included periodic acid–Schiff (PAS) and Luxol Fast Blue (LFB). The manipulation, fixation and brain sampling were made following the recently published methodology (Sacchini et al., 2022) applied by our group since 2009. In addition, a subset of samples (including skin, lung, kidney, liver, spleen, prescapular lymph node and brain) were stored at −80 °C for microbiological and virological analysis. For molecular viral screening, 0.5 g of each fresh frozen tissue sample was processed as previously described (Arbelo et al., 2012). Genomic DNA and RNA was simultaneously extracted by pressure filtration (QuickGene DNA tissue kit S, Kurabo, Japan), following the manufacturer's instructions with slight modifications (Sacristán et al., 2015). Molecular analyses for *cetacean morbillivirus* (CeMV) and herpesvirus detection were performed as previously reported (Felipe-Jiménez et al., 2022; Felipe-Jiménez et al., 2021).

In order to identify the genus and the species of the trematodes, a quantitative Polymerase Chain Reaction (q-PCR) based on SYBR® Green technique (Mini Opticon Real-Time PCR System. MJ Mini Personal Thermal Cycler of BIO RAD) was performed as previously published (Sierra et al., 2020). Two pairs of primers were designed, one of these amplifying a 230 bp nucleotide sequence of the NADH dehydrogenase, subunit 3 (ND3) gene of *Nasitrema delphini* (GenBank Accession no.

KT180216) (Fraija-Fernández et al., 2016): (Forward: 5′- CGG ATT GGT TTT CGT TGT CT −3′; Reverse: 5′- ACC CAA CCT AAG CAA GAG CA −3′), and the other set to amplify a 241 bp nucleotide sequence of the 18S rRNA gene, partial of *Nasitrema globicephalae* (GenBank Accession no. AJ004968) (Fernandez et al., 1998b): (Forward: 5′- CTG AAT CGG TGG GTT TGT CT −3′; Reverse: 5′- CCG TCT GTC CCT CTT AAC CA −3′). Two negative controls (for extraction and amplification) and an amplification-positive control were included (*Nasitrema* sp. from the brain tissue sample of a bottlenose dolphin previously obtained in our laboratory). The PCR product was purified using a Real Clean spin kit (REAL®, Durviz, s.l., Valencia, Spain) and sequenced (Sanger method). A BLAST search (BLAST: Basic Local Alignment Search Tool, 2021) was conducted to confirm the identity of the PCR amplicon, which was compared with other somewhat similar sequences published in GenBank. A set of closely related sequences were aligned using ClustalW algorithm through software MEGA X (Kumar et al., 2018). The phylogenetic tree was constructed using the Maximum Likelihood Method and the Hasegawa-Kishino-Yano model with a discrete Gamma distribution to model the evolutionary rate differences among sites (5 categories (+G, parameter = 0.3452)). To determine the reliability of the tree was used the Bootstrap method with 500 replicates.

The *Nasitrema* sp. obtained sequences from the NADH dehydrogenase, subunit 3 (ND3) gene, were identified from this study and were deposited in GenBank under accession no. OP480061.

Additionally, 54 beaked whales were reviewed in detail, representing the 60.7% of the total number ($n = 89$) of strandings involving BWs along the Canary coasts between January 1999 and December 2017, according to our database. The selection criteria were that all these specimens were subjected to a complete standardized necropsy and currently preserving recollected samples. Specifically, 35 animals were Cuvier's BWs (*Ziphius cavirostris*) and 19 specimens belonged to the *Mesoplodon* genus: 1 True's BW (*Mesoplodon mirus*), 2 Sowerby's BWs (*Mesoplodon bidens*), 6 Blainville's BWs (*Mesoplodon densirostris*) and 10 Gervais' BWs (*Mesoplodon europaeus*). All the information about the life history data (species, age category, sex, length weight, stranding date, location and stage), the decomposition stage, body condition (good, moderate, poor or emaciated) (Arbelo et al., 2013; Díaz-Delgado et al., 2018), and the reproductive status (mature or immature is registered and summarized in Supplementary Table 1. Postmortem examination included the careful gross and microscopic examination of the cephalic region (nasal air sacs system, pterygoid sinus, tympano-periotic complex and central nervous system (CNS)) as well as the respiratory tract (tracheobronchial tree and lungs) including evaluation of the presence of parasites.

3. Results

Gross examination of the CET 824 revealed a very poor body condition (814 kg weight) characterized by extremely concave dorsal profile with visible costal reliefs, and serous atrophy of fat. The thickness of blubber was measured in three positions, (dorsal, lateral, and ventral) in a skin sample from the cranial zone of the dorsal flipper: 3.4 centimetres (cm), 3 cm and 3.2 cm. In addition, no food remains were observed in the digestive tract. Moderate multifocal linear-bleeding and linear erosive skin lesions were mainly found in the flippers, rostrum, and ventral parts of the body, which were associated with a suspected active stranding event. Moderate and multifocal congestion and haemorrhages were also shown in the lung and mediastinal lymph nodes, acoustic fat and melon; although the most outstanding finding was brain lesions. More in detail, serial coronal sections of the brain revealed the presence of multifocal, tortuous, irregular, well-defined, yellow-green areas of soft texture, mainly located in the frontal lobe of the right cerebral hemisphere extending from the inner part of the white matter to the adjacent dorso-lateral cortex without affecting the meninges (Fig. 1 A). Similar lesions were also present in the diencephalon (Fig. 1 B).

Histologically, multiple areas of necrosis surrounded by pyogranulomatous inflammation (with pigment laden macrophages and scattered multinucleated giant cells), as well as multifocal haemorrhages associated with an intralesional adult trematode (Fig. 2 A, B and C) were observed. The trematode was 2 mm wide in a transversal cut (not evident grossly during the sampling of the brain) with a thin tegument where no spines were observed (probably lost post-mortem) surrounded by spongy parenchyma where no other structures were recognizable (because of necrosis) except the vitellaria (Fig. 2 C). Refringent yellow triangular to oval-shape eggs of about 50 to 75 μm in diameter were found in the cortex and diencephalon. The eggs were found in the same lesion area but not near the adult parasite (Fig. 2 D). Other histopathological changes observed in the CNS were diffuse gliosis, multifocal

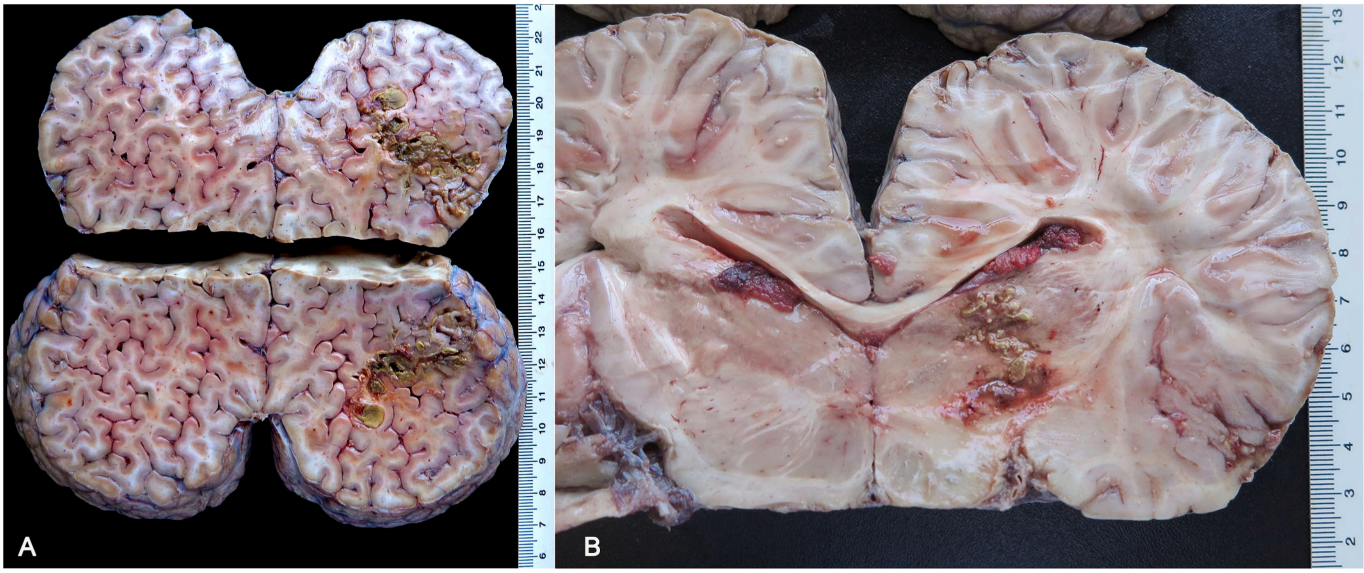

Fig. 1. Gross findings in the brain from a Blainville beaked whale (CET 824): coronal sections (post fixation in formalin). A) Sections from the frontal lobe of the right cerebral hemisphere showed a severe chronic-active multifocal pyogranulomatous and necrotizing encephalitis. B) The diencephalon section also presented multifocal, irregular, well-demarcated and elevated yellow-green lesions, surrounded by a hemorrhagic halo, which are compatible with the aberrant parasitic migratory route. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

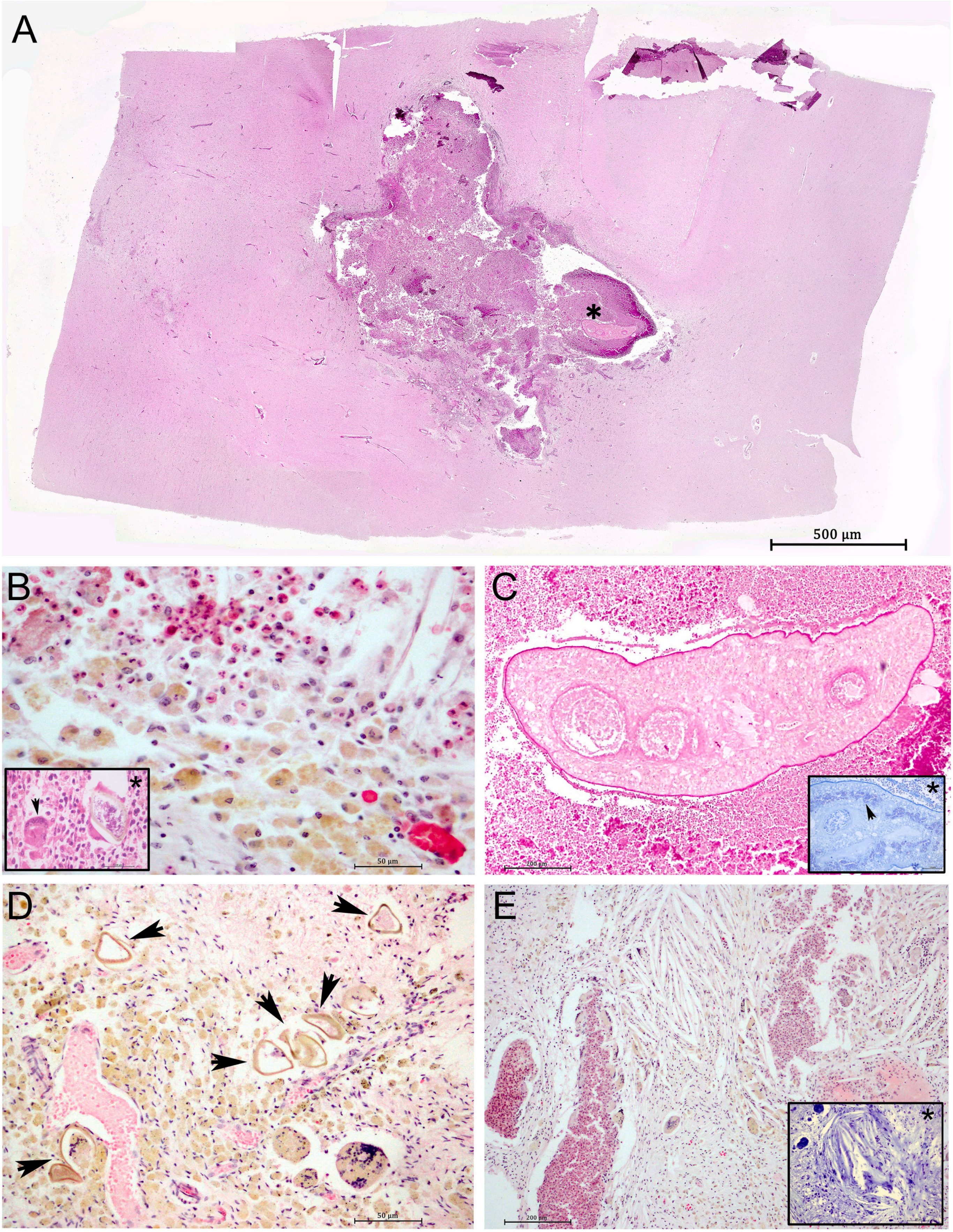

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Fig. 2. Histopathology: brain findings from a Blainville beaked whale (CET 824). A) Histological overview showing an area of necrosis and pyogranulomatous inflammatory reaction associated with an intralesional adult trematode (asterisk) at the diencephalon. PAS stain, low-power magnification. B) Detail of the inflammatory infiltrate mainly composed of neutrophils and pigment laden macrophages cells. HE stain, high-power magnification Inset (*): Detail of a multinucleated giant cell (arrow). HE stain, high-power magnification. C) A closer view showing the only one adult *Nasitrema* sp. found at the histopathological examination. The trematode was surrounded by necrosis, macrophages and polymorphonuclear cells. HE stain, medium-power magnification. Inset (*): Detail of the trematode's internal structures compatible with vitellaria (arrow). LFB stain, medium-power magnification. D) Triangular to oval-shape refringent yellow of trematode eggs (arrows) surrounded by macrophages with intracytoplasmic yellowish pigment. HE stain, high-power magnification. E) Multifocal cholesterol clefts associated with the presence of *Nasitrema* sp. HE stain, medium-power magnification. Inset (*): cholesterol clefts stained with LFB stain, medium-power magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

lymphoplasmacytic perivascular cuffs, perivascular edema and multifocal presence of cholesterol clefts (Fig. 2 E). The liver presented lymphoplasmacytic multifocal pericholangitis and periductal fibrosis associated with the presence of adult flukes and eggs, compatible with trematodes species belonging to the *Brachycladiidae* family. Mild multifocal granulomatous fasciitis and panniculitis with intralesional degenerating metazoan cuticle (compatible with *Crassicauda* sp.), multiorgan moderate haemorrhages (lymph nodes, acoustic fat and melon), mild multifocal lymphoplasmacytic myocarditis, as well as mild-moderate multifocal acute degenerative changes in the skeletal (hypercontracted myofibers and segmental necrosis) and cardiac muscle (juxtanuclear vacuolization and increased acidophilic cytoplasm of the myocardiocytes), and mild multifocal lymphoplasmacytic gastroenteritis and adrenalitis were observed.

A partial sequence of the ND3 gene (152 base pairs in length excluding primers) was obtained from brain sample of CET 824, which presented the highest percentage of similarity, (99.34% with a 99% Query cover (QC)) with a *N. delphini* sequence (GenBank Accession no. KT180216) detected in a common dolphin stranded in Spain (Canary Islands) (Fraija-Fernández et al., 2016). The sequence also showed 91.33% of homology (99% QC) with a *N. attenuatum* sequence (GenBank Accession no. MN264280) obtained from a Guiana dolphin stranded in Brazil, and 90.07% of homology (99% QC) with a *N. globicephalae* sequence (GenBank Accession no. AF034557) from a long-finned pilot whale stranded in Chile (Fernandez et al., 1998a). The phylogenetic tree analysis involved ten nucleotide sequences (Fig. 3). The tree was divided into two branches, one of them contained five *Nasitrema* genus sequences and the other is formed by five sequences from several trematodes belonging to the *Brachycladiidae* family which also constitute the root of the tree. The phylogenetic analysis showed that the sequence obtained from the Blainville's BW of our study is clustered, with a bootstrap value of 79, with the only available sequence of *N. delphini* (KT180216) detected in the common dolphin stranded in the same geographic area. The branches were condensed with a bootstrap value over 50%. All the tested samples from CET 824 were CeMV negative by RT-qPCR (Felipe-Jiménez et al., 2022). However, Herpesvirus DNA was detected by conventional PCR in the kidney, liver, prescapular lymph node and brain tissue samples with no associated lesions. (Felipe-Jiménez et al., 2021).

Evidence of the presence of these parasites was not found in any of the other BWs from our study for which the following anatomical regions were grossly inspected: the respiratory track (100%), the pterygoid sinus (40.7%), the nasal air sacs (12.9%), the tympano-periotic complex (11.1%), and the CNS (55.5%). Some of these regions were also microscopically analysed; the brain (55.5%), the respiratory track (100%), and the tympano-periotic complex (7.4%). This information is compiled in Table 2. Notwithstanding, some of the microscopic findings displayed by brain sample of CET 824 were also observed in animal identified as CET 770; specifically, a localized mild granulomatous meningitis with pigment-laden macrophages was noticed. However, this case was negative for *Nasitrema* spp. by the two q-PCRs.

4. Discussion

We report the presence of a platyhelminth parasite, identified as *N. delphini*, in a BW stranded in the Canary Islands. Evidence of the presence of this parasite was not found in any of the revised 54 beaked whales. Thus, the presence of *Nasitrema* sp. detected in one BW stranded in the Canary Islands represents the 0.2% of the animals in which at least three regions (lungs, pterygoid sinuses and CNS) were consistently examined (1/20). The fluke was associated with brain lesions that could have been responsible for the stranding and/or death of the animal. In the recent years, an increased number of neuropathological findings has been reported to be related to cetaceans stranding (Arbelo et al., 2013; Díaz-Delgado et al., 2018; Pintore et al., 2018; St. Leger et al., 2018), from which infectious and inflammatory conditions of the CNS are among the most common leading natural causes of stranding and death. A wide spectrum of pathogens (viruses, bacteria, fungi and parasites) has been reported affecting the CNS of these marine mammals, which can result in fatal acute, subacute, or chronic meningitis and/or encephalitis (Pintore et al., 2018; Sierra et al., 2022; Sierra et al., 2020). Parasitic meningoencephalitis has been more frequently related to protozoans (*Toxoplasma gondii*) (Gulland et al., 2018; St. Leger et al., 2018), while helminthic brain lesions are less frequently described and usually involve necrotizing encephalitis along the migratory path in which intralesional nematodes and trematodes (adults, eggs, and/or

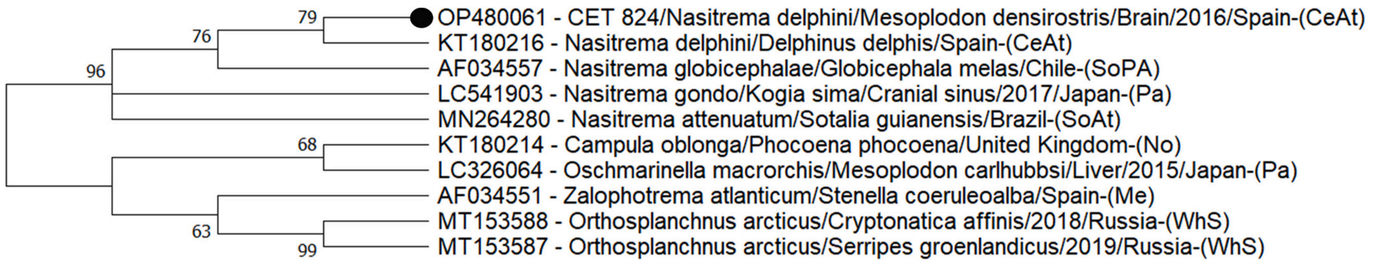

Fig. 3. Maximum likelihood phylogenetic tree for the nucleotide partial sequences of the ND3 gene. The phylogenetic tree consists of 10 sequences from reported cases of *Nasitrema* spp. and trematodes belonging to the *Brachycladiidae* family. The Neighbour-Join and BioNJ algorithms together with the Hasegawa-Kishino-Yano model and Gamma distribution to model the evolutionary rate differences among sites [5 categories (+G, parameter = 0.3452)], were chosen to construct the tree. The Bootstrap method was performed to resample 500 replicates and evaluate the reliability of the tree. The branches were condensed with a bootstrap value over 50%. The accession number from GenBank, the trematode species from the *Brachycladiidae* family, the host, the date of collection, and the geographic area of stranding were used to identify the nucleotide sequences. Abbreviations: CeAt (Central Atlantic Ocean); Me (Mediterranean Sea); No (North Sea); Pa (Pacific Ocean); SoAt (South Atlantic Ocean); SoPA (South Pacific Ocean); WhS (White Sea).

Table 2
Summary of the study's anatomical regions examined for detecting *Nasitrema* spp. in the beaked whales of our study. Notes. G: gross examination; M: microscopic examination; (−): not examined; A: absence of parasites; P: presence of parasites.

		Respiratory tract		Pterygoid sinus	Nasal air sac system	Tympano-periotic complex		CNS	
ID CODE	SPECIES	G	M	G	G	G	M	G	M
CET 86	*Ziphius cavirostris*	A	A	–	–	–	–	A	A
CET 103	*Ziphius cavirostris*	A	A	A	–	–	–	A	A
CET 108	*Ziphius cavirostris*	A	A	A	–	–	–	–	–
CET 113	*Ziphius cavirostris*	A	A	–	–	–	–	–	–
CET 134	*Mesoplodon europaeus*	A	A	–	–	–	–	–	–
CET 180	*Mesoplodon densirostris*	A	A	A	–	–	–	A	A
CET 181	*Ziphius cavirostris*	A	A	A	–	–	–	A	A
CET 182	*Ziphius cavirostris*	A	A	A	–	–	–	A	A
CET 183	*Ziphius cavirostris*	A	A	A	–	–	–	A	A
CET 184	*Ziphius cavirostris*	A	A	A	–	–	–	A	A
CET 185	*Mesoplodon europaeus*	A	A	A	–	–	–	A	A
CET 189	*Ziphius cavirostris*	A	A	–	–	–	–	–	–
CET 213	*Mesoplodon densirostris*	A	A	–	–	–	–	A	A
CET 236	*Ziphius cavirostris*	A	A	–	–	A	A	A	A
CET 243	*Mesoplodon densirostris*	A	A	–	–	–	–	A	A
CET 259	*Mesoplodon europaeus*	A	A	–	–	–	–	A	A
CET 264	*Ziphius cavirostris*	A	A	–	–	–	–	–	–
CET 265	*Ziphius cavirostris*	A	A	–	–	–	–	–	–
CET 294	*Ziphius cavirostris*	A	A	–	–	–	–	–	–
CET 304	*Ziphius cavirostris*	A	A	–	–	–	–	A	A
CET 322	*Ziphius cavirostris*	A	A	–	–	–	–	–	–
CET 333	*Mesoplodon europaeus*	A	A	–	A	–	–	A	A
CET 334	*Mesoplodon europaeus*	A	A	–	–	–	–	A	A
CET 338	*Mesoplodon europaeus*	A	A	A	–	A	A	A	A
CET 352	*Ziphius cavirostris*	A	A	–	–	–	–	A	A
CET 354	*Mesoplodon europaeus*	A	A	–	–	A	–	–	–
CET 379	*Mesoplodon bidens*	A	A	A	–	–	–	A	A
CET 471	*Ziphius cavirostris*	A	A	–	–	–	–	A	A
CET 503	*Ziphius cavirostris*	A	A	–	–	–	–	–	–
CET 510	*Mesoplodon europaeus*	A	A	A	–	–	–	A	A
CET 547	*Mesoplodon europaeus*	A	A	A	–	–	–	A	A
CET 576	*Ziphius cavirostris*	A	A	A	–	–	–	A	A
CET 579	*Ziphius cavirostris*	A	A	–	–	–	–	–	–
CET 591	*Ziphius cavirostris*	A	A	–	A	–	–	–	–
CET 593	*Ziphius cavirostris*	A	A	–	–	–	–	–	–
CET 620	*Ziphius cavirostris*	A	A	–	–	–	–	–	–
CET 624	*Ziphius cavirostris*	A	A	–	–	–	–	–	–
CET 631	*Mesoplodon europaeus*	A	A	A	–	–	–	A	A
CET 636	*Mesoplodon mirus*	A	A	A	–	–	–	A	A
CET 645	*Ziphius cavirostris*	A	A	–	A	–	–	–	–
CET 680	*Ziphius cavirostris*	A	A	–	A	–	–	–	–
CET 688	*Ziphius cavirostris*	A	A	–	–	–	–	–	–
CET 695	*Mesoplodon densirostris*	A	A	A	–	–	–	A	A
CET 711	*Mesoplodon densirostris*	A	A	–	–	–	–		–
CET 712	*Ziphius cavirostris*	A	A	–	–	–	–	–	–
CET 719	*Ziphius cavirostris*	A	A	–	–	–	–	–	–
CET 720	*Ziphius cavirostris*	A	A	A	A	–	–	–	–
CET 770	*Ziphius cavirostris*	A	A	A	A	A	A	A	A
CET 771	*Ziphius cavirostris*	A	A	A	–	–	–	A	A
CET 818	*Ziphius cavirostris*	A	A	–	–	–	–	–	–
CET 824	*Mesoplodon densirostris*	A	A	A	A	A	A	A	**P**
CET 827	*Mesoplodon bidens*	A	A	A	–	A	A	A	A
CET 833	*Ziphius cavirostris*	A	A	A	–	–	–	–	–
CET 852	*Mesoplodon densirostris*	A	A	A	A	A	–	A	A
CET 855	*Ziphius cavirostris*	A	A	–	–	–	–	A	A

larvae) can be found (Summers et al., 1995; Vandevelde et al., 2012).

The presence of adults and/or eggs of *Nasitrema* spp. have been previously identified in odontocete cetacean brain lesions, which typically are described as grossly multiple, irregularly, brownish areas with cavitation and necrosis, and thickened meninges (Cowan et al., 1986; Dailey, 1985; Dailey and Walker, 1978; Degollada et al., 2002; Forrester, 1991; Lewis and Berry, 1988; O'Shea et al., 1991; Phillips and Suepaul, 2017; Ridgway and Murray, 1972; Sierra et al., 2020). Similar lesions were grossly observed in the animal from our study. Histologically, liquefactive necrotic abscessed areas bordered by diffuse infiltrates of macrophages with occasional hemosiderophages, giant cells, gliosis and infiltrative lymphoplasmacytic or fibrinopurulent meningoencephalitis have been observed associated to the presence of eggs and/or adult worms of these flukes (Degollada et al., 2002; Díaz-Delgado et al., 2018; Lewis and Berry, 1988; O'Shea et al., 1991; Phillips and Suepaul, 2017; Ridgway and Murray, 1972; Sierra et al., 2020); although the participation of *Nasitrema* spp. in chronic nervous lesions, such as non-suppurative leptomeningitis, cannot be ruled out (Degollada et al., 2002). Similar lesions were found in the animal from our study in addition to the presence of cholesterol clefts within the necrotic tissue, which have been less frequently described in brain nasitremiasis (Ridgway and Murray, 1972). We only noticed brain lesions during the histopathological examination in two out of the 30 brain microscopically examined (6.7%), although these lesions could only be associated to the presence of *Nasitrema* sp. in one of these brains. Despite the life cycle of *Nasitrema* spp. remains unknown (Howard, 1983; Lewis and

Berry, 1988), brain lesions are thought to be the result of an aberrant migration of the parasite through the VIII cranial nerve (Cowan et al., 1986; Howard, 1983; Lewis and Berry, 1988; Morimitsu et al., 1992; Ridgway and Murray, 1972). In odontocetes, this nerve is externalized, extending from the brain stem to the retro-bullar space of the tympanoperiotic complex without an osseous cover (Cozzi et al., 2017; Ketten, 1992; Morimitsu et al., 1992; Morimitsu et al., 1987; Morimitsu et al., 1986; Reysenbach de Haan, 1957). In spite of that the head and air sacs are the most common anatomic locations where these parasites inhabit (Arbelo et al., 2013; Cowan et al., 1986; Dailey and Ridgway, 1976; Dailey and Walker, 1978; Degollada et al., 2002; Díaz-Delgado et al., 2018; Ebert and Valente, 2013; Forrester, 1991; Kikuchi et al., 1987; Kumar et al., 1975; Lewis and Berry, 1988; Morimitsu et al., 1986, 1987, 1992; Neiland et al., 1970; O’Shea et al., 1991; Oliveira et al., 2011; Phillips and Suepaul, 2017; Ridgway and Murray, 1972; Schwab, 1985; Shiozaki and Amano, 2017; Sierra et al., 2020; Walker and Cowan, 1981), any trematode was found neither in the inner ear, the middle ear, the VIII cranial nerve, the pterygoids’ sinus or the cranial sacs neither in animal CET 824 nor in other BW including in this study. Thus, in accordance, it remains unsolved, how the parasite reached the brain in the animal of our study.

Incoordination, loss of equilibrium, and echolocation dysfunction are some of the clinical signs exhibited by the affected animals by brain nasitremiasis, highlighting the possible role of this trematode parasite in the stranding events due to alterations in echolocation and balance orientation (Dailey and Walker, 1978; Lewis and Berry, 1988; Morimitsu et al., 1992; Morimitsu et al., 1987; Morimitsu et al., 1986; O’Shea et al., 1991). In that sense, despite being found death, a live stranding event was suspected in the animal from our study according to the extremely freshness of the carcass and several necropsy findings, such as skin erosive lesions, congestion of acoustic submandibular fat and melon and mild-moderate acute myodegenerative changes in cardiac muscle, as previously described (Herráez et al., 2013; Herráez et al., 2007). The severe lesions of the brain, produced by the widely distributed nasitremiasis, might could affect the foraging and diving pattern of the animal and potentially cause its stranding.

The animal of our study displayed an extremely poor body condition and herpesvirus co-infection, which could indicate a debilitated and immunocompromised state. *Nasitrema* spp. co-infections with other pathogens have been previously reported in cetaceans: in a captive Atlantic bottlenose dolphin with a bacterial and mycotic saculitis (Wright et al., 1979), in a striped dolphin with pneumonia produced by *Vibrio damsela* (O’Shea et al., 1991), in a Pacific white-side dolphin with *Edwardsiella tarda* septicaemia (Lewis and Berry, 1988), and in a melon-headed whale from which *Edwardsiella* sp. was recovered from purulent material of brain lesions (Lewis and Berry, 1988; Phillips and Suepaul, 2017).

Different species within the genus *Nasitrema* have been detected in a wide range of odontocete cetacean (Table 3). In our study, the parasitic identification mostly relied on the molecular and phylogenetic analyses, as most of the internal parasitic structures were indistinguishable. The remaining morphohistological features displayed by the parasite from our study (specifically the size of the adult fluke and eggs) are in concordance with those of different species of the genus *Nasitrema;* specifically, *dalli*, *delphini*, *globicephalae* (Ebert and Valente, 2013; Kikuchi et al., 1987; Neiland et al., 1970) and *attenuata* (Kumar et al., 1975; Lim et al., 2016). However, the sequence obtained from this study presented the highest nucleotide similarity with a sequence of *N. delphini* detected in a common dolphin stranded in the same geographic area as the animal from our study (Canary Islands) (Fraija-Fernández et al., 2016). The presence of the *N. delphini* had been additionally reported in two common bottlenose dolphins also stranded in the Canary Islands (morphologically or molecularly identified) (Díaz-Delgado et al., 2018; NIH: National Library of Medicine; National Center for Biotechnology Information, 2021). Apart from this geographical area, this species within the genus *Nasitrema* had been only morphologically identified in the common dolphin species from southern California (Dailey and Walker, 1978; Neiland et al., 1970).

Table 3
Summary of the detected *Nasitrema* species in cetaceans.

Nasitrema spp.	Cetacean host	References
N. delphini	Bottlenose dolphin	(Díaz-Delgado et al., 2018; NIH: National Library of Medicine; National Center for Biotechnology Information, 2021)
	Common dolphin	(Dailey and Walker, 1978; Fraija-Fernández et al., 2016; Neiland et al., 1970)
N. globicephalae	Common dolphin	(Dailey and Walker, 1978)
	Long-finned pilot whale	(Fernandez et al., 1998a; Fraija-Fernández et al., 2016)
	Rough-toothed dolphin	(Ebert and Valente, 2013)
	Striped dolphin	(Oliveira et al., 2011)
	Pantropical spotted dolphin	(Oliveira et al., 2011)
	Short-finned pilot whale	(Neiland et al., 1970)
	False killer whale	(Neiland et al., 1970)
	Pacific white-side dolphin	(Dailey and Walker, 1978)
	Northern right whale dolphin	(Dailey and Walker, 1978; Neiland et al., 1970)
N. attenuata	Long-beaked common dolphin	(Lim et al., 2016)
	Indo-Pacific Bottlenose Dolphin (*Tursiops aduncus*)	(Kleinertz et al., 2014)
	Guiana dolphin	(Ebert and Valente, 2013)
	Rough-toothed dolphin	(Ebert and Valente, 2013)
	False killer whale	(Neiland et al., 1970)
	Bottlenose dolphin	(Kumar et al., 1975)
N. gondo	Melon-headed whale	(Morimitsu et al., 1986)
	Long-finned pilot whale	(Morimitsu et al., 1987; Yamaguti, 1951)
	False killer whale	(Morimitsu et al., 1987)
	Risso’s Dolphin	(Morimitsu et al., 1992)
N. spathulatum	Narrow-ridged finless porpoise	(Shiozaki and Amano, 2017)
	Finless porpoise	(Ozaki, 1935)
N. sunameri	Narrow-ridged finless porpoise	(Shiozaki and Amano, 2017)
	Finless porpoise	(Yamaguti, 1951)
N. lanceolata	Long-finned pilot whale	(Neiland et al., 1970)
N. lagenorhynchus	Pacific white-side dolphin	(Kikuchi et al., 1987)
N. dalli	Dall’s porpoise	(Dailey and Walker, 1978; Yamaguti, 1951)
N. stenosoma	Common dolphin	(Dailey and Walker, 1978)

5. Conclusion

Nasitremiasis have been reported in several cetacean species, with the presence of intralesional trematodes in lungs, pterygoids sinus, middle and inner ear, VIII cranial nerve and brain. Although this is, to the authors´ best knowledge, the first description of *Nasitrema* sp. (molecularly identified as *N. delphini*) in a member of the *Ziphiidae* family (namely, a Blainville ‘s BW).

Due to the severe clinical signs (incoordination, loss of equilibrium, and echolocation dysfunction) that may be derived from tissues damage caused by the presence of adult flukes or their eggs in the cephalic region, a careful postmortem diagnostic (including a deep inspection of the cranial structures) is needed to increase the potential diagnostic of the disease.

Additionally, further investigation is required to a better understanding of the biology, epidemiology, and pathogenesis of *Nasitrema* spp. in BWs and other cetacean species.

Institutional review board statement

The necropsy of the animal from this study was possible thanks to the permission for the management of stranded cetaceans conceded by the Spanish Ministry of the Environment (SGPM/BDM/AUTSPP/70/2019). Furthermore, neither animal was sacrificed and not experiments were performed with live animals, so ethical review and approval were waived.

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CRediT authorship contribution statement

Idaira Felipe-Jiménez: Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Antonio Fernández:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision, Funding acquisition. **Yara Bernaldo de Quirós:** Methodology, Formal analysis. **Marina Arregui-Gil:** Methodology, Formal analysis. **Raquel Puig-Lozano:** Methodology, Formal analysis. **Eva Sierra:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The *Nasitrema delphini* obtained sequence from a Blainville beaked whale (*Mesoplodon densirostris*) stranded in the Canary Islands has been deposited in GenBank (under accession number: OP480061).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2023.03.009.

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6. CONCLUSIONES

Zifio de True (*Mesoplodon mirus*). FOTOGRAFÍA: John Horsfall

6. CONCLUSIONES

1. El uso de técnicas moleculares ha permitido detectar material genético de herpesvirus y *Morbillivirus de los cetáceos* en el 16,4% de los zifios analizados (n=55) varados en el archipiélago canario en un periodo de 19 años (1999-2017).

2. La prevalencia de herpesvirus en los zifios varados analizados en las Islas Canarias para el periodo de estudio fue del 14,5% de los animales y del 5,1% de los tejidos, con una mayor proporción de muestras positivas en pulmón y riñón.

3. Se ha determinado, por primera vez, herpesvirus en dos especies de la familia *Ziphiidae*, el zifio de Gervais (*Mesoplodon europaeus*) y el zifio de Sowerby (*Mesoplodon bidens*).

4. En tres de los ocho animales positivos a herpesvirus se pudo asociar la positividad obtenida con lesiones a nivel histopatológico, en concreto dos casos de nefritis tubulointersticial necrotizante con cuerpos de inclusión intranucleares en dos zifios de Blainville (*Mesoplodon densirostris*) y un caso de linfadenitis y esplenitis necrótica con cuerpos de inclusión intranucleares en células monocíticas en un zifio de Cuvier (*Zipius cavirostris*).

5. Se han detectado tres secuencias noveles de *alphaherpesvirus* en zifios del género *Mesoplodon*: zifio de Blainville, zifio de Gervais y zifio de Sowerby. El resto de las secuencias detectadas en los zifios varados de nuestro estudio muestran una mayor proximidad a muestras detectadas en otras especies de zifios y a la especie delfín listado (*Stenella coeruleoalba*) de áreas geográficas próximas, sugiriendo que interacciones interespecíficas entre estas cuatro especies de zifios analizadas en nuestro estudio podría explicar la transmisión de herpesvirus dentro del familia *Ziphiidae*, así como la transmisión de herpesvirus entre distintas especies de cetáceos de regiones cercanas.

6. La prevalencia de *morbillivirus de los cetáceos* en los zifios varados analizados (n=55) en las islas canarias para el periodo de estudio (1999-2017) fue del 1,8% de los animales y del 0,3% de los tejidos, en concreto en una muestra de piel sin lesiones asociadas a nivel histopatológico.

7. Se ha registrado la detección más temprana (en el año 2002) de una secuencia de *morbillivirus del delfín* en la especie zifio de Cuvier.

8. Se ha demostrado que la secuencia parcial del gen de la fosfoproteína del morbillivirus del delfín detectada en nuestro estudio presentaba una mayor similitud con otra secuencia detectada en un delfín listado varado en las Islas Canarias el mismo año, lo que apoya la hipótesis previa de la existencia de una circulación endémica de una cepa del *morbillivirus de los delfines* en el Océano Atlántico; así como de la infección cruzada entre especies distintas de cetáceos.

9. Se ha descrito una nasitremiasis cerebral, por la especie *Nasitrema delphini,* en una hembra adulta de zifio de Blainville, constituyendo la primera descripción en un miembro de la familia *Ziphiidae*.

7. REFERENCIAS

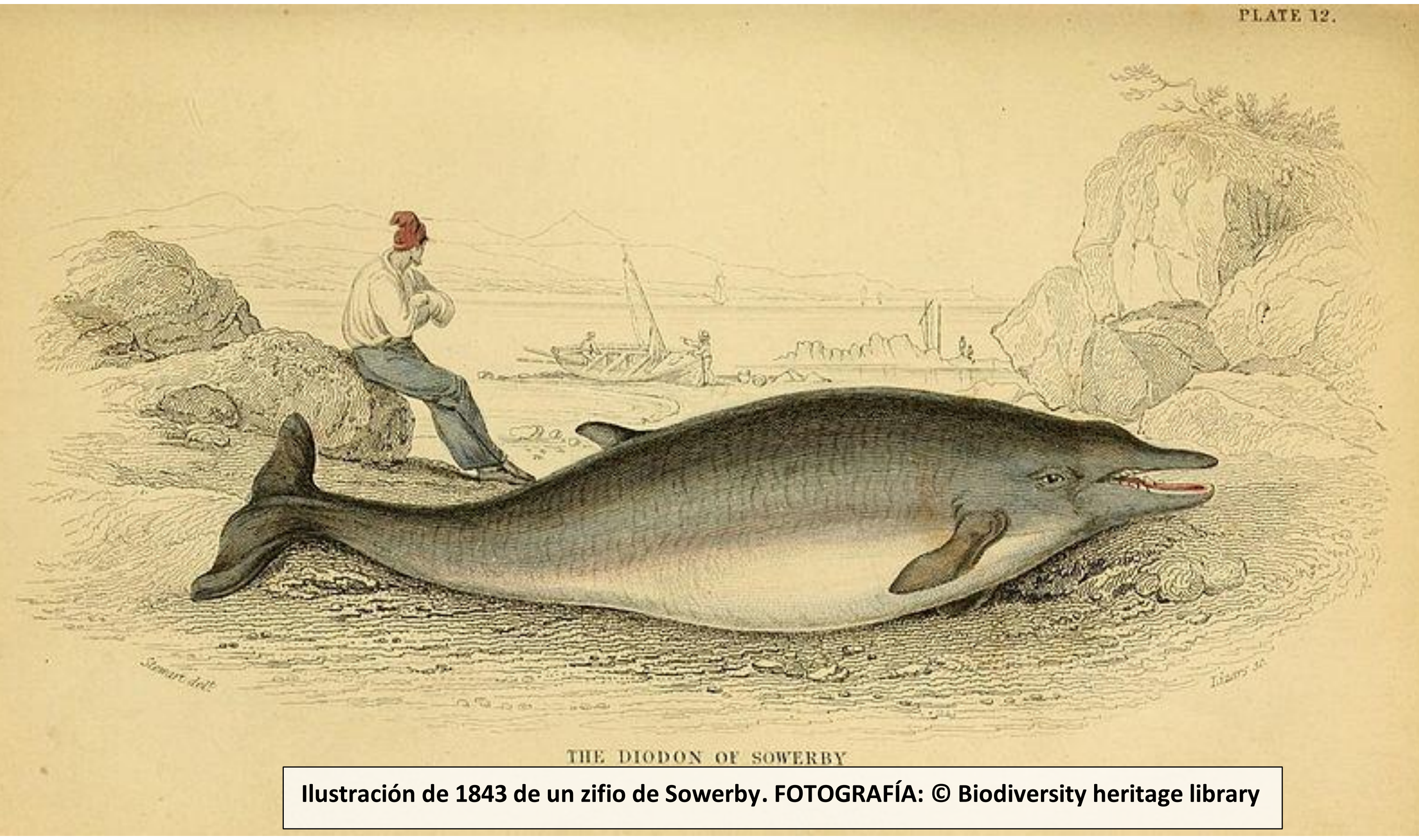

Ilustración de 1843 de un zifio de Sowerby. FOTOGRAFÍA: © Biodiversity heritage library

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