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CASE REPORT

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First description of a lesion in the upper digestive mucosa associated with a novel gammaherpesvirus in a striped dolphin (*Stenella coeruleoalba*) stranded in the Western Mediterranean Sea

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Abstract

Background A wide variety of lesions have been associated with herpesvirus in cetaceans. However, descriptions of herpesvirus infections in the digestive system of cetaceans are scarce.

Case report A young female striped dolphin stranded in the Valencian Community (Spain) on the 6th August 2021. The animal showed external macroscopic lesions suggestive of an aggressive interaction with bottlenose dolphins (rake marks in the epidermis). Internally, the main findings included congestion of the central nervous system and multiple, well-defined, whitish, irregularly shaped, proliferative lesions on the oropharyngeal and laryngopharyngeal mucosa. Histopathology revealed lymphoplasmacytic and histiocytic meningoencephalitis, consistent with neuro brucellosis. The oropharyngeal and laryngopharyngeal plaques were comprised histologically of focally extensive epithelial hyperplasia. As part of the health surveillance program tissue samples were tested for cetacean morbillivirus using a real-time reverse transcription-PCR, for *Brucella* spp. using a real-time PCR, and for herpesvirus using a conventional nested PCR. All samples were negative for cetacean morbillivirus; molecular positivity for *Brucella* spp. was obtained in pharyngeal tonsils and cerebrospinal fluid; herpesvirus was detected in a proliferative lesion in the upper digestive mucosa. Phylogenetic analysis showed that the herpesvirus sequence was included in the *Gammaherpesvirinae* subfamily. This novel sequence showed the greatest identity with other Herpesvirus sequences detected in skin, pharyngeal and genital lesions in five different species.

Conclusions To the best of the authors' knowledge, this is the first report of a proliferative lesion in the upper digestive mucosa associated with gammaherpesvirus posititvity in a striped dolphin (*Stenella coeruleoalba*).

Keywords Herpesvirus, Proliferative lesion, Upper digestive tract, Oropharynx, Laryngopharix, *Brucella*, Transmission, Immunosuppression

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Background

Herpesvirus (HV) infections have been observed in mammals, birds, reptiles, fish, amphibians, and bivalves [1]. *Herpesviridae* family consists of three subfamilies: *Alpha-*, *Beta-*, and *Gammaherpesvirinae* [2]. Cetacean herpesvirus strains are usually classified according to the partial nucleotide se quence of a locus of their DNA polymerase (DNApol) gene [3]. So far, only *Alpha-* and/or *Gammaherpesvirinae* have been identified in eight cetacean families: Delphinidae, Kogiidae, Ziphiidae, Physeteridae, Monodontidae, Phocoenidae, Iniidae (odontocetes), and Balaenopteridae (mysticetes) [4–10].

HV can establish latent infection, during which no viral particles are produced [11, 12], and revert to an active replication under stress or immunosuppression [13]. Interestingly, HV also can cause immunosuppression in humans [14, 15], and in other animal species [16–18], including cetaceans [5]. For this reason, it is common to find coinfections with other pathogens, such as Cetacean Morbillivirus (CeMV) [19–22] and *Brucella* spp [20, 22].

In cetaceans, HV molecular positivity has been associated with or without pathological findings [19, 23, 24]. Specifically, Gammaherpesvirus (GHV) positivity have been associated with cutaneous and mucosal lesions, mainly from genital mucosa [4, 6, 19, 24–32]. Recently, GHV infections have also been detected in the Central Nervous System of cetaceans [10, 19–21, 24], although its significance still needs to be clarified [19–21, 24].

Although numerous cases of HV infection have been described in cetaceans in recent years, very few data is available concerning its detection in the digestive system of marine mammals [19]. Therefore, the description of HV-related lesions in the digestive tract extends our knowledge about HV in cetaceans and raises relevant questions about their transmission and significance. To the best of the authors' knowledge, this is the first report of a proliferative lesion in the oropharyngeal and laryngopharyngeal mucosa associated with a GHV in a striped dolphin (*Stenella coeruleoalba*).

Case presentation

On 6th August 2021, a juvenile male striped dolphin stranded alive at the shoreline of Pobla de Farnals, Valencian Community, Spain (39° 34' 40" N, 0° 19' 34" W), presenting difficulty maintaining balance and swimming in circles. The veterinary staff from Fundación Oceanogràfic, which is part of the Regional Stranding Network, carried out a complete veterinary examination including blood sampling. The animal was stabilized with emergency medication and the Rose Bengal Test (RBT) was performed for brucellosis screening in field conditions. Due to the animal condition, with neurological clinical signs, and a positive result in the RBT, the dolphin was humanely euthanized, and a full necropsy was performed the same day, according to previous literature [33, 34]. The main macroscopic lesions included longitudinal, parallel and shallow lacerations (rake marks) in the epidermis of the caudal fin, head, back and abdominal region (Fig. 1.A and 1.B); diffuse hyper pigmented dotting on the skin of the dorsal area of the thoracic region, cranial to the dorsal fin (Fig. 1.C); multifocal oval lesions with irregular borders, and heterogeneous hypo-hyperpigmented colour, in the left thoracic region (Fig. 1.D); oval lesion, approximately 4 cm in diameter, with a hypo pigmented centre on the ventral part of the animal (Fig. 1.E); presence of two specimens of *Xenobalanus* spp. on dorsal fin and right pectoral fin; emphysema in the central region of the left lung, with the presence of congestive areas

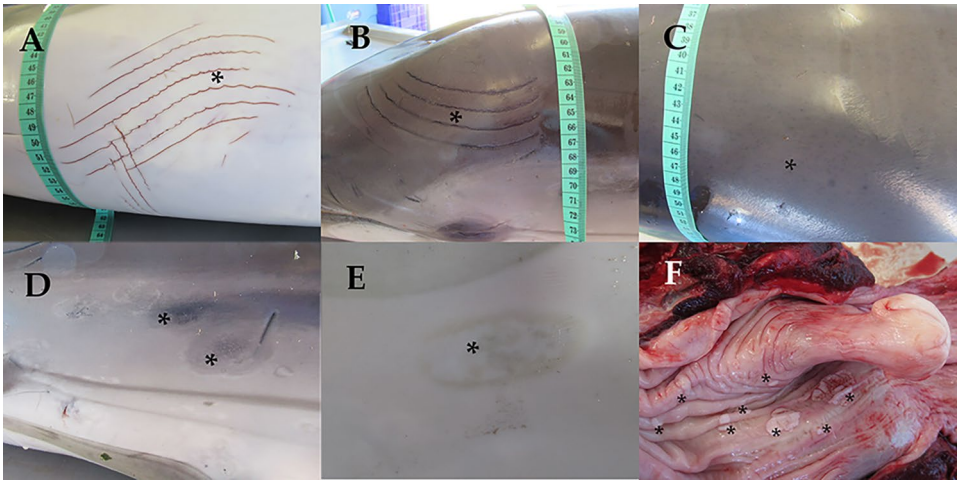

Fig. 1 Most relevant macroscopic findings. The lesions described are highlighted by asterisks (*). **(A)** Rake marks in the abdominal region. **(B)** Rake marks in the head. **(C)** Diffuse hyper pigmented dotted lesion in the dorsal area of the thoracic region. **(D)** Oval lesions with irregular borders and hypo-hyper pigmented heterogeneous coloration in the left thoracic region. **(E)** Oval lesion with a hypo pigmented centre on the ventral part of the animal. **(F)** Oropharyngeal and laryngopharyngeal mucosa with multiple well-defined, whitish, irregularly shaped proliferative lesions (plaques)

medially and atelectasis in the most caudo-ventral area; hemorrhage with the presence of a clot in the middle dorsal zone of the left lung; moderate presence of free serosanguinous fluid in the pleural cavity; multiple, well-defined, whitish, raised, irregularly shaped, 0.3 to 4 cm, proliferative plaques on the oropharyngeal and laryngopharyngeal mucosa, adjacent to the glottis (Fig. 1.F); congestion, hemorrhage and edema in the pre scapular and pulmonary lymph nodes; and moderate congestion in the meninges.

Representative samples from several organs were collected during necropsy, including: normal skin, skin lesions, blubber, muscle, upper digestive mucosa with plaques, thyroid gland, adrenal gland, kidney, liver, pancreas, spleen, thymus, pharyngeal tonsils, pre scapular lymph node, mesenteric lymph node, lung, heart, spinal cord, cerebrum, cerebellum, cerebrospinal fluid (CSF) and serum.

Two sets of tissue samples were collected: the first set was preserved in 10% neutral buffered formalin and used for conventional histopathology, while the second set was stored at −80 °C and used for molecular analysis.

Histologically, the mucosa of the oropharyngeal and laryngopharyngeal plaques was thickened with over 80 layers piled over a folded and tortuous basement membrane. Basal cells were piled and disorganized with slightly basophilic cytoplasm and frequent mitotic figures. Middle layers contained mildly swollen cells with occasional perinuclear vacuolation. Superficial layers of approximately one third of the mucosal width were comprised of flattened cells with brightly eosinophilic cytoplasm and condensed, hyperchromatic, occasionally fragmented nuclei (Fig. 2A and B). Rare cells immediately underneath the hyper eosinophilic layers had a swollen nucleus with marginated hyperchromatic chromatin and a central pale eosinophilic inclusion (Fig. 2C).

Other histological findings, in order of significance, were: severe, chronic multifocal to coalescing, lymphoplasmacytic and histiocytic meningoencephalitis in the brainstem; confluent multifocal granulomatous pancreatic ductitis with intralesional trematodes; mild to moderate, chronic, confluent multifocal granulomatous pneumonia with intralesional 400 micron cross sections of nematodes with a celomic cavity, thick cuticle, celomyarian musculature, lateral cords, large intestine, consistent with metastrongyles (*Halocercus* sp.); subacute, multifocal, pulmonary lymph node sinus haemorrhage with erythrophagocytosis; mild, multifocal, suppurative and granulomatous cholangitis and multifocal bile stasis; granulomatous and eosinophilic mesenteric lymphadenitis; and moderate, multifocal, subcapsular renal haemorrhage.

For molecular diagnosis, tissue samples were evaluated for the presence of herpesvirus, CeMV and *Brucella* spp., as part of the health surveillance program carried out in the Valencian Community. In addition, the presence of poxvirus in skin lesions was ruled out. Standard precautions were taken during all laboratory procedures to avoid cross-contamination of samples. All samples were diluted 1:10 with phosphate-buffered saline (PBS) and homogenized using stainless steel 4.8-mm beads (Next Advance, New York, USA). Total DNA and RNA from the homogenates was extracted using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics), based on the manufacturer's instructions.

The molecular diagnosis of the herpesvirus was carried out based on a previously described pan-herpesvirus nested PCR targeting the DNApol gene [35]. This assay was applied to the extracted DNA from skin, skin lesions, lesion in the upper digestive mucosa, pharyngeal tonsils, prescapular lymph node, cerebrum and cerebellum. Only the lesion lesion in the upper digestive mucosa was found positive. The 212-bp amplicon was purified using the QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany), and sequenced by Sanger sequencing. The obtained nucleotide sequence was submitted to Genbank and attributed the accession number OP716694.The herpesvirus nucleotide sequence was analyzed phylogenetically using the maximum likelihood method in MEGA X software [36] (Fig. 3). For this phylogenetic study, we included three human herpesvirus sequences, one canid herpesvirus sequence, one bovine herpesvirus sequence, one phocine herpesvirus sequence, one caprine herpesvirus sequence, and 33 cetacean herpesvirus sequences

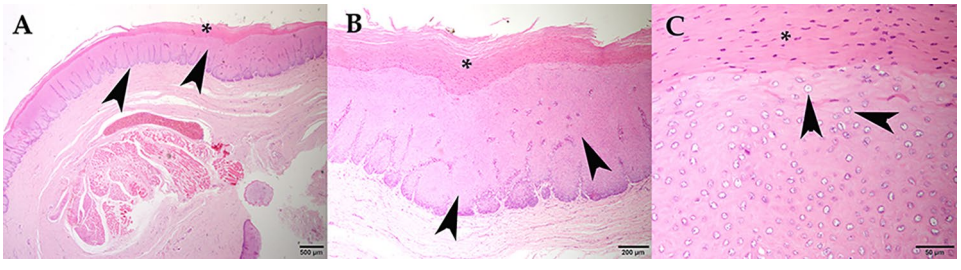

Fig. 2 Raised plaque in the upper digestive mucosa. **A and B.** Epithelial hyperplasia (arrow) showing numerous layers piled over a folded and tortuous basement membrane and brightly eosinophilic, retained superficial layers (*). **C.** Detail of the superficial layers with hyperchromatic and occasionally fragmented nuclei (*) and underlying layers containing rare cells with marginated chromatin and a central, pale eosinophilic inclusion (arrow)

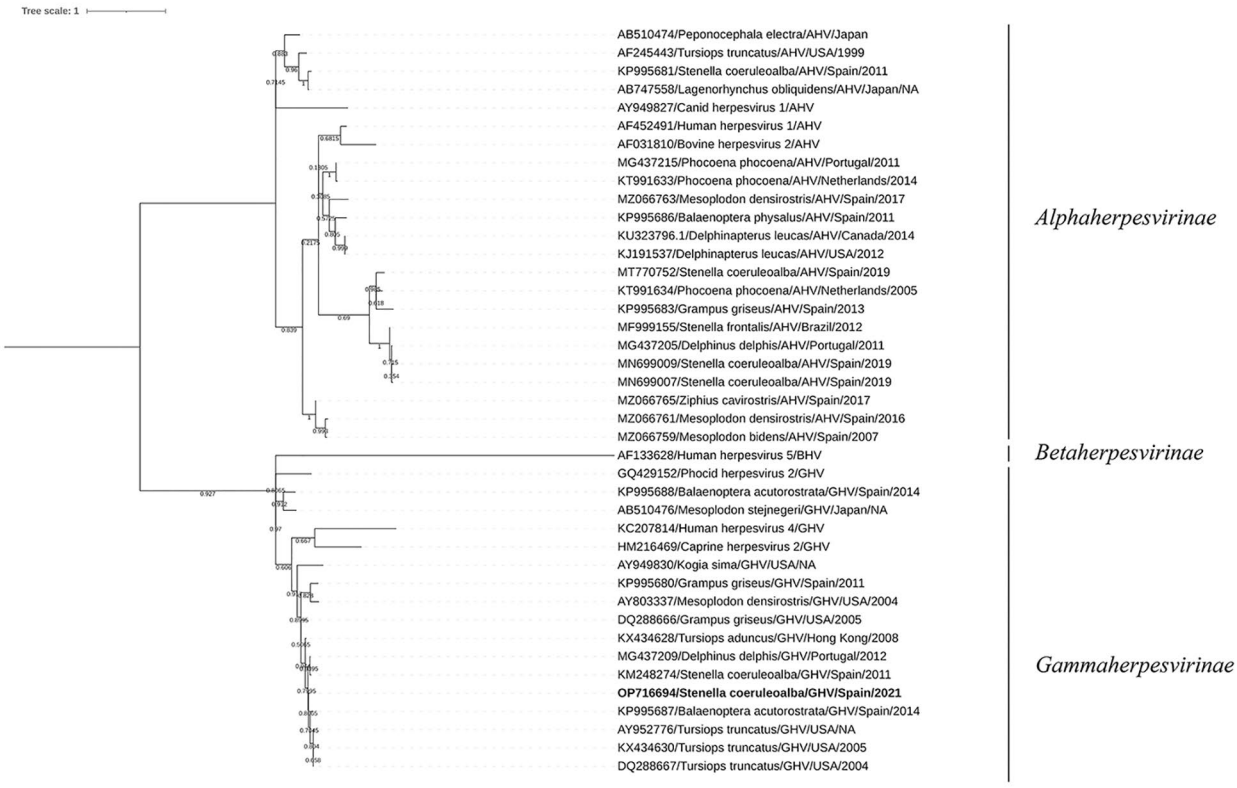

Fig. 3 Maximum-likelihood phylogram of herpesviruses, based on partial nucleotide sequence of the DNApol gene. Nucleotide sequences of cetacean HV are named according to accession number, host species, subfamily of herpesvirus, country of origin and year of isolation. Sequence identified in the present study has been highlighted in bold and with a black square. Abbreviations: AHV Alphaherpesvirus, GHV Gammaherpesvirus.

(including both odontocetes and mysticetes) detected in different in countries worldwide since 1999, in addition to the sequence described in this study. The accuracy of the sequence alignments was corrected in order to confer with the acceptance threshold value (<0.8) for average p-distance [37, 38]. A bootstrap analysis with 2000 replicates was used to test the reliability of the tree. The resulting tree was rooted and edited with iTOL editor [39]. Nucleotide identities were calculated based on the p-distance (1−p-distance)*100.

The phylogenetic analysis revealed that sequence OP716694 belong to the *Gammaherpesvirinae* subfamily and was part of a clade with a GHV from a striped dolphin from Spain (KM248274), a GHV from a short-beaked common dolphin (*Delphinus delphis*) from Portugal (MG437209), a GHV from a common minke whale (*Balaenoptera acutorostrata*) from Spain (KP995687), and GHV from bottlenose dolphins (*Tursiops truncatus*) from the USA (DQ288667, AY952776, KX434630).

Our sequence (OP716694) showed the highest nucleotide identity (98.13%) with GenBank sequence DQ288667, obtained from a penile lesion of a bottlenose dolphin from the USA. It also showed an identity higher than 90% with the following GenBank sequences: MG437209 (95.45%), obtained from a genital lesion of a short-beaked common dolphin from Portugal; KM248274 and KX434630 (93.94%), obtained from a lesion on the penis of a striped dolphin from Spain, and from a lesion on the pharyngeal mucosa of a bottlenose dolphin from the USA, respectively; KP995687 (93.55%), obtained from the skin of a common minke whale from Spain; KX434628 (93.46%), obtained from a penile lesion of an Indo-Pacific bottlenose dolphin (*Tursiops aduncus*) from Hong Kong; and AY952776 (92,42%), obtained from a penile lesion of a bottlenose dolphin from the USA.

RNA extracted from cerebrum, cerebellum, prescapular lymph node, lung, and pharyngeal tonsils were tested for CeMV using a reverse transcription-real time PCR assay targeting the fusion protein gene and is based on the Universal Probe Library platform [40]. All samples tested negative.

For *Brucella* spp. molecular detection was carried, following a previously described real-time PCR, targeting the IS711 insertion sequence [41], was performed on the following tissue samples: lung, pharyngeal tonsils, cerebrum, and CSF. The authors reported a lower limit of detection for this assay of 0.2-2 fg, depending on the Brucella species analyzed [41]. Since a negative result was

considered when the Cycle threshold (Ct) was greater than 40, molecular positivity was observed in pharyngeal tonsils (Ct: 39.43) and CSF (Ct: 32.95). The PCR products from positive reactions were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany), following manufacturer's instructions. The amplicons (178 bp [41]), were directly sequenced by Sanger DNA sequencing, which confirmed the positive result.

Additionally, the DNA from the skin lesions (n=3) was tested for Poxvirus by conventional PCR, targeting DNApol gene [42]. However, molecular positivity was not detected.

Discussion and conclusions

Detailed and reliable knowledge about the diseases and infectious agents that can affect cetaceans may provide useful insights into marine mammals' health [19]. In this report, post-mortem analysis of a stranded striped dolphin and various routine molecular diagnosis were carried out to assess the lesions that the animal presented, as well as to implement a sanitary surveillance of the main infectious agents. This surveillance allowed us to associate a novel GHV sequence (OP716694) to a lesion in the oropharyngeal and laryngopharyngeal mucosa.

Other proliferative lesions in the oropharynx mucosa associated to GHV have been recently described in six rough-toothed dolphins (*Steno bredanensis*) from the Atlantic coast of the USA [43] and a Risso's Dolphin (*Grampus griseus*) from the Mediterranean Spanish Coast [19]. GHV infections have been previously identified in hyperplastic skin and mucosal lesions, mainly genital [4, 9, 19, 24–27, 32, 44]. In line with this, the results of the phylogenetic analysis showed that the novel GHV sequence detected in this study was included in the GHV group and shared high identity with other GenBank sequences detected in skin (KP995687), pharyngeal (KX434630), and genital (DQ288667, MG437209, KM248274, KX434628, AY952776) lesions.

The infection of the upper digestive mucosa by a GHV sequence that shares significant identity with several genital sequences raises questions about its origin. Interestingly in a Risso's dolphin from Spain, similar hyperplastic plaques were identified in both esophageal and genital mucosa, but they were associated with different HV subfamilies: GHV and Alphaherpesvirus (AHV), respectively [19]. Other similar GHV-associated esophageal lesions were detected in multiple rough-toothed dolphins of the same pod, suggesting that horizontal transmission may also exist [43]. Considering that in this case it was a juvenile dolphin, and that the present sequence shared 98.13% homology with the GenBank sequence DQ288667 detected in the genital mucosa, it is not possible to rule out suckling as a route of transmission since the proximity of the mammary slits to the genital slit in cetaceans. Other possible routes of infection involving orogenital contact include socio-sexual behaviors, such as 'goosing', in which an individual moves its rostrum into the genital area of another [45]. However, the role of water as a mechanical transmitter cannot be excluded either, as has been suggested for other HV [46, 47]. On the other hand, aerial transmission through desquamated infected cells from oropharyngeal lesions could be also a possibility. Respiratory and gastrointestinal systems are in close contact, [48] and this fact could imply that the GHV located in the esophageal mucosa could be excreted upon exhalation, and be potentially transmitted through the respiratory route.

Regarding the pathological significance of these oropharyngeal and laryngopharyngeal lesions, further research should be done in order to elucidate the transmission routes of the GHV that may be the cause of these lesions, as well as their impact on the health of the cetacean population.

Concerning the stranding cause, this dolphin had erratic swimming, probably due to the lymphoplasmacytic and histiocytic meningoencephalitis. This clinical sign, the location and type of lesion as well as the inflammatory infiltrate were consistent with neurobrucellosis [49]. Positive RBT and molecular analysis confirmed the *Brucella* spp. infection. Although bacterial culture is considered the 'gold standard' technique for the diagnosis of brucellosis, real-time PCR has also been previously performed to confirm Brucella infections in cetaceans [50, 51]. On the other hand, hemorrhages in the internal organs, the presence of the rake marks in the epidermis, and the separation of the parallel lacerations (interdental space) were suggestive of an aggressive interaction from bottlenose dolphins, as previously described in striped dolphins in this area [52, 53]. However, well-defined borders and the absence of fibrous tissue are indicative of an acute or subacute process. The other lesions were mainly associated with parasitic migrations.

It is not possible to conclude whether GHV infection occurred before or after *Brucella* infection. However, this individual exhibited signs of chronic illness, including poor body condition. Parasitic infestation, coupled with chronic brucellosis, are consistent with immunosuppression. It is well known that HV can establish latent infections [11, 12] and revert to an actively replicating state when an immunosuppressive or stress event occurs [13]. Therefore, it is likely that immunosuppression could have favored the appearance of this lesion or played a role. However, future studies which take these variables into account will need to be undertaken to better understand the pathogenesis of these lesions.

In conclusion, to our knowledge, we described for the first time a GHV-associated proliferative lesion in the upper digestive mucosa of a striped dolphin and we

submitted the novel sequence to GenBank for future studies. The current findings enhance our understanding of herpesvirus pathology in cetaceans raising relevant questions about their transmission and significance.

Abbreviations

HV	Herpesvirus
DNApol	DNA polymerase
AHV	Alphaherpesvirus
GHV	Gammaherpesvirus
RBT	Rose Bengal Test
CSF	Cerebrospinal Fluid
CeMV	Cetacean Morbillivirus
Ct	Cycle threshold

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Author contributions
IVC wrote the manuscript, and JLCP, MAJM, VMC, MMB, DGP and JMSV helped revise it. JLCP, VMC and MMB performed necropsies and collected samples. IVC performed molecular analysis and performed phylogenetic study. MAJM contributed by performing the histopathology analysis. DGP and JMSV coordinated and reviewed data collection, data analysis and manuscript writing. All authors read and approved the final manuscript.

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Data Availability
DNA sequence is available in the GenBank with following accession number: OP716694.

Declarations

Ethics approval and consent to participate
The Oceanogràfic Foundation is part of the Stranding Network thanks to an agreement between the "Ciudad de las Artes y las Ciencias" and the "Conselleria de Infraestructuras, Territorio y Medio Ambiente" by which the rights of veterinary assistance are transferred to the Oceanogràfic Foundation in cases of stranded sea turtles and cetaceans. This agreement includes the rights to euthanize animals when necessary, perform necropsies in collaboration with the University of Valencia, and the collection of samples from the carcasses of stranded cetaceans. In the collection of post-mortem tissues for research purposes, the approval of the corresponding ethics committee is not required. Additionally, the field study did not involve endangered or protected species.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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