FACULTAT DE VETERINÀRIA DE BARCELONA

"Morbillivirus infection in Mediterranean striped dolphins (*Stenella coeruleoalba*) during the 2007 epidemic and the post-epidemic years"

Tesis doctoral presentada por Sara Soto Martín para acceder al grado de Doctor en Veterinaria dentro del programa de Doctorado en Medicina y Sanidad Animal de la Facultad de Veterinaria de la Universidad Autónoma de Barcelona, bajo la dirección del Dr. Mariano Domingo Álvarez

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Certifica:

que la memoria titulada **"Morbillivirus infection in Mediterranean striped dolphins (*Stenella coeruleoalba*) during the 2007 epidemic and the post-epidemic years"**, presentada por Sara Soto Martín para la obtención del grado de Doctor en Veterinaria, se ha realizado bajo su dirección en la Universidad Autónoma de Barcelona.

Y para que conste a todos los efectos oportunos, firmo la presente en Bellaterra, 30 de Junio de 2014.

Dr. Mariano Domingo Álvarez

Para Gor, Pau y Jana

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TABLE OF CONTENTS

SUMMARY / RESUMEN

In the summer 2007 high number of striped dolphins (*Stenella coeruleoalba*) stranded on the Spanish Mediterranean coast. Histopathological, immunohistochemical and RT-PCR analysis performed with dolphin material from these strandings confirmed the re-emergence of a morbillivirus epizootic, which afterwards spread to French Mediterranean waters. The Dolphin morbillivirus (DMV, a Cetacean Morbillivirus (CeMV) viral strain) detected showed close relationship to the DMV strain isolated in the previous epizootic, occurred in 1990. This thesis aimed to compile three articles published in peer-reviewed journals and one book chapter describing the pathological findings observed in the striped dolphins affected by the 2007 epizootic in Catalonia, and the pathological and RT-PCR findings observed in Catalonia, Valencia Region and Andalusia in the following years. The lesions observed in the dolphins affected by the 2007 epizootic were similar to those described in the 1990 epizootic, with bronchiolo-interstitial pneumonia, lymphoid depletion and non-suppurative meningoencephalitis as main microscopic findings. In addition several dolphins affected in 2007 also showed, as occurred in 1990, secondary opportunistic infections. But beside to *Toxoplasma gondii* and *Aspergillus* spp. like fungus, alphaherpesvirus systemic concomitant infection and disease were also observed in the 2007 outbreak. Striped dolphins affected in 2007 were shorter in length than those affected in 1990, suggesting that older dolphins may have been protected from the infection due to a previous exposure to the virus in the first epizootic. The immunohistochemical studies performed with cetacean tissues from strandings occurred on the Catalonian coast between the 1990 and the 2007 morbillivirus epizootics would support the hypothesis that CeMV has not circulated in the Mediterranean Sea in the interepidemic period. In the years following the 2007 epizootic numerous striped dolphins have been randomly found along the Spanish Mediterranean coast showing the chronic form of morbillivirus infection already described after the 1990 epizootic. The chronic form is characterized by the detection of lesions and virus only in the brain of the affected dolphins, instead of the classic systemic distribution of lesions and virus observed during the epizootics. This presentation of the disease in dolphins shows similarities with other chronic diseases caused by morbilliviruses, as subacute sclerosing panencephalitis due to Measles virus in humans, and old dog encephalitis due to Canine distemper virus in dogs. However, further investigations would be required in order to clarify the pathogenesis and the viral persistence mechanisms in the dolphin morbillivirus chronic form.

En el verano de 2007 un elevado número de delfines listados (*Stenella coeruleoalba*) apareció varado en la costa Mediterránea en España. Los estudios histopatológicos, inmunohistoquímicos y de RT-PCR realizados a partir de muestras de estos delfines confirmaron la reaparición de una epidemia de morbillivirus, que se extendió posteriormente por el Mediterráneo Francés. En dichas muestras se detectó un Morbillivirus del delfín (DMV, considerado una cepa de la especie Morbillivirus de cetáceos CeMV) muy próximo al virus detectado en la epidemia anterior, ocurrida en 1990. Esta tesis reune tres artículos publicados en revistas científicas y un capítulo de un libro, en los que se describen los hallazgos patológicos en delfines listados varados durante la epidemia en 2007 en Cataluña, y los hallazgos patológicos y de RT-PCR en los delfines listados varados en Cataluña, Comunidad Valenciana y Andalucía en los años posteriores. Los delfines afectados por la epidemia de 2007 mostraron lesiones similares a las descritas en la epidemia de 1990, siendo neumonía bronquiolo-intersticial, depleción linfoide y meningoencefalitis no supurativa los hallazgos microscópicos principales. También en 2007 algunos delfines presentaban lesiones asociadas a agentes infecciosos oportunistas entre los que se encontraba, además de *Toxoplasma gondii* y hongos compatibles con *Aspergillus* spp., alfaherpesvirus. Este virus ha sido detectado en varios delfines afectados durante la epidemia, causando en un caso enfermedad concomitante a la infección por morbillivirus. Los delfines listados muertos durante la epidemia de 2007 mostraron menor longitud que los muertos en la epidemia de 1990, lo que podría sugerir que los delfines de mayor edad que estuvieron en contacto con el virus en 1990 estarían protegidos frente a una reinfección. Los estudios inmunohistoquímicos realizados con tejidos de cetáceos varados en Cataluña en los años transcurridos entre ambas epidemias respaldarían la hipótesis de ausencia de circulación del CeMV en el Mediterráneo en el periodo interepidémico. En los años posteriores a 2007 numerosos delfines listados han sido hallados esporádicamente en la costa Mediterránea española afectados por la forma crónica de la infección de morbillivirus. Esta presentación crónica de la enfermedad, descrita tras la epidemia de 1990, se caracteriza por la deteccción de lesiones y virus únicamente en el encéfalo de los delfines, a diferencia de la presencia de lesiones y virus en múltiples órganos que muestran los delfines afectados durante las epidemias. La forma crónica de morbillivirus en delfines muestra similitudes con otras infecciones crónicas debidas a morbillivirus, como la panencefalitis esclerosante subaguda causada por el virus del sarampión en humanos, y la encefalitis del perro viejo causada por el virus del moquillo canino. Sin embargo, dado que actualmente son más las incógnitas que las respuestas, se requeriría continuar investigando sobre el morbillivirus del delfín para poder aclarar aspectos como la patogenia y los mecanismos de persistencia del virus en los delfines afectados por la forma crónica.

INTRODUCTION

Barcelona is an amazing city that has the Mediterranean Sea as part of its numerous attractions. In this Sea, among numerous marine species, thousands of dolphins swim and live. I was so lucky to perform the four years of my specialization in veterinary pathology in the Universitat Autònoma de Barcelona, where I could collaborate in the pathologic investigations performed with dolphins found stranded on the Catalonian coast, and with dolphin samples sent to our diagnostic service from Andalusia and Valencia Region. This PhD work encompasses three scientific articles published in international peer-reviewed journals, and one book chapter. In the appendix of the thesis, unpublished related results included in the discussion chapter are also described. All this work is focused in the effects and consequences of morbillivirus infection in Mediterranean striped dolphins (*Stenella coeruleoalba*), by far the main infectious disease affecting this species in the Mediterranean Sea.

1. Morbilliviruses in marine mammals

Morbilliviruses (family Paramyxoviridae, order Mononegavirales) are enveloped single-stranded negative-sense RNA viruses of about 16kb in length. Measles virus (MV), Canine distemper virus (CDV), Rinderpest virus (RPV) and Peste-des-petits-ruminants virus (PPRV) are the species classically included in this group. It was in the late 80's when, for the first time, morbillivirus infections affecting marine mammals were discovered. In 1987-1988 CDV caused high mortality of Baikal seals (*Phoca sibirica*) in the Russian Baikal lake (Grachev et al. 1989). In 1988 a new morbillivirus named Phocine distemper virus (PDV) was recognised as the agent killing thousands of harbour seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*) at the North and Baltic Sea (Osterhaus & Vedder 1988, Kennedy, Smyth, McCullough et al. 1988, Mahy et al. 1988, Osterhaus 1989). Morbillivirus infection in cetaceans was also recognized for the first time in 1988 affecting harbour porpoises (*Phocoena phocoena*) in the Irish Sea. This virus was designated as Porpoise morbillivirus (PMV) (Kennedy, Smyth, Cush et al. 1988, Kennedy et al. 1991). Dolphin morbillivirus (DMV) in 1990 in the Mediterranean Sea, and Pilot whale morbillivirus (PWMV) in 2000 in the North Atlantic Ocean, were discovered afterwards as the cause of mass mortality in striped dolphins (*Stenella coeruleoalba*), and the cause of long-finned pilot whales (*Globicephala melas*) death, respectively (Domingo et al. 1990, Domingo et al. 1992,

Taubenberger et al. 2000). PMV, DMV and PWMV are actually considered strains of a unique viral species named Cetacean morbillivirus (CeMV) (King et al. 2012). DMV and PMV have been found in dolphins, while PWMV and DMV have been found in pilot whales (Taubenberger et al. 2000, Fernández et al. 2008). In porpoises only PMV has been detected (Kennedy et al. 1998). Recently other CeMV variants divergent from the known strains have been proposed, detected in cetaceans found in the Brazilian coast and the western Australian coast (Groch et al. 2014, Stephens et al. 2014).

Morbilliviruses are distributed worldwide in marine mammals, with high number of pinniped and cetacean species infected (Barrett et al. 1995, Kennedy 1998, Van Bressem et al. 1999, 2001). Marine mammal populations can suffer high mortality due to morbillivirus infection when the virus enters into a naïve population (Kennedy, Smyth, McCullough et al. 1988, Domingo et al. 1990), or single/discrete mortality events (Daoust et al. 1993, Wohlsein, Puff et al. 2007). Re-emergence of morbillivirus epizootics have been described in both seals and cetaceans (Jensen et al. 2002, Raga et al. 2008, Noaa fisheries 2014), with a build-up of susceptible individuals in the populations along the years following the first epizootic. The origin of a morbillivirus mortal event in a marine mammal population is not elucidated in the majority of cases. Harp seals (*Phoca groenlandica*) are likely PDV reservoirs, and their migratory movements have been related with European northern seal epizootics (Dietz et al. 1989, Kennedy 1998, Härkönen et al. 2006). Pilot whales have been also hypothesized as CeMV vectors for Mediterranean dolphins (Van Bressem et al. 2009).

Morbillivirus infection in cetaceans and pinnipeds causes a systemic process mainly affecting the lungs, the lymphoid organs and the brain. Although clinical signs and macroscopic lesions have been described, the diagnosis of morbilliviral disease in marine mammals is based in the observation of specific microscopic lesions in target organs, demonstration of morbilliviral antigen and/or viral nucleic acid in tissues as well as isolation of the virus (Kennedy et al. 1989, Domingo et al. 1992, Barrett et al. 1993, Nielsen et al. 2008). The classical triad of microscopic lesions in affected cetaceans and pinnipeds is: bronchiolo-interstitial pneumonia, loss and necrosis of lymphoid cells in lymphoid organs and non-suppurative meningoencephalitis. Nuclear and/or cytoplasmic viral inclusion bodies and multinucleate syncytial cells are usual associated microscopic findings (Baker 1992, Domingo et al. 1992, Duignan et al. 1992, Kennedy 1998, Fernández et al. 2008).

A more extensive review of morbillivirus infection in marine mammals can be found in the following chapter of the thesis, in the publication entitled “Morbillliviruses in sea mammals”.

2. Morbillivirus infection in the Mediterranean Sea

The Mediterranean Sea (figure 1) is considered as one of the most important spots in the world regarding biodiversity. It is divided into the western and the eastern Mediterranean, connected through the strait of Sicily. Twenty-one cetacean species have been sighted in the Mediterranean waters, eight of these as resident populations: bottlenose dolphin (*Tursiops truncatus*), Couvier´s beaked whale (*Ziphius cavirostris*), fin whale (*Balaenoptera physalus*), long-finned pilot whale, Risso´s dolphin (*Grampus griseus*), short-beaked common dolphin (*Delphinus delphis*), sperm whale (*Physeter macrocephalus*) and striped dolphin. The Mediterranean Sea also hosts one pinniped species, the Mediterranean monk seal (*Monachus monachus*) (Otero & Conigliaro 2012).

Figure 1: Mediterranean Sea political map

The striped dolphin is the most abundant cetacean species in the Mediterranean, in both the western and the eastern areas. It is particularly common in the Ligurian Sea, Gulf of Lyon, the

Alboran Sea and the waters between the Balearic Islands and the Iberian Peninsula. The IUCN red list (International Union for Conservation of Nature and Natural Resources) includes this species in the list of "vulnerable" regarding the Mediterranean status (Otero & Conigliaro 2012). In 1991 the western Mediterranean striped dolphin population was estimated in 117.880 individuals, excluding the Tyrrhenian Sea (95% CI= 68.379-214.800) (Forcada et al. 1994). The population of striped dolphins in the central Mediterranean Spanish waters was estimated during the period of 2001 to 2003 in 15.778 individuals (95% CI= 10.940 to 22.756) by line transect sampling, and 16.892 individuals (CV=0.16) by spatial modelling methods (Gómez de Segura et al. 2006, Gómez de Segura et al. 2007).

From 1990 to 1992 thousands of striped dolphins died in the Mediterranean Sea due to a morbillivirus infection (Domingo et al. 1990, 1992, Duignan et al. 1992). The morbillivirus isolated from these individuals was a new morbillivirus strain, close to the Porpoise morbillivirus (PMV) discovered in porpoises in the late 80's (Kennedy, Smyth, Cush et al. 1988), and was designated as Dolphin morbillivirus (DMV) (Barrett et al. 1993, Visser, Van Bressem, De Swart, et al. 1993). DMV is considered today a viral strain included in the viral species Cetacean Morbillivirus (CeMV) (King et al. 2012). The first dolphins documented to have died due to this viral infection were found stranded in Valencia (Spanish coast) in July 1990. However later investigations confirmed dolphins which had died before this date in southern Spanish Mediterranean coasts (Domingo et al. 1992). The extension of the CeMV infection from the Atlantic Ocean to the Mediterranean Sea due to infected marine mammals was hypothesized (Domingo et al. 1992, Visa i Esteve 1994). The infection spread through the Spanish Mediterranean coast, arriving at the French coast in September. From there, spreading eastward in a wave-like moving, the epizootic arrived at the Italian coast in June 1991, and at the Greek coast a couple of months later. In the summer 1992 the epizootic had extended to the Turkish Mediterranean coast (Aguilar & Raga 1993, Visser, Van Bressem, Barrett, et al. 1993, Visa i Esteve 1994, Domingo et al. 1995). In 1994 the mortality of common dolphins in the Black Sea (*Delphinus delphis ponticus*) abnormally increased also due to morbillivirus infection, acquired from their Mediterranean striped dolphin neighbours (Birkun et al. 1999). As the infection reached new areas, the previous affected coasts began to subside it. In Catalonia, the last dolphin affected by the epizootic was recorded at the end of October 1990 (Domingo et al. 1995).

Fifteen years later, in the summer of 2007, a second morbillivirus epizootic affecting striped dolphins occurred in Spanish Mediterranean waters. The infection was detected in July in

Valencia, spreading then to southern and northern Spanish coasts and to France (Raga et al. 2008, Keck et al. 2010). The virus found in the affected dolphins was very closely related to the virus that caused the epizootic in 1990 (Raga et al. 2008). In addition it was also almost identical to the virus reported as the cause of death of long-finned pilot whales found along the southern Spanish Mediterranean coast and the Balearic Islands only several months before, from October 2006 to April 2007 (Fernández et al. 2008), and to the virus reported as the cause of death of a bottlenose dolphin in the Northeast Atlantic Ocean (Canary Islands) in July 2005 (Sierra, Zucca et al. 2014). This second epizootic resembled the previous morbillivirus outbreak in the Mediterranean Sea, both causing important mortality in the striped dolphin Mediterranean population and both beginning on the Spanish coast in the early summer. However, they differed in four main aspects: a) the 1990 epizootic affected a very large area of the Mediterranean Sea, reaching the Turkish coast and even the Black Sea, while the 2007 epizootic was limited to the western Mediterranean; b) the 2007 epizootic was milder, with hundreds instead of thousands of dolphins affected; c) during the 2007 epizootic the affected dolphins frequently showed shorter length, meaning younger age, than the dolphins affected in the 1990 epizootic; d) while in the 1990 epizootic only striped dolphins were found to have died due to morbillivirus infection, in the 2007 epizootic also a bottlenose dolphin and pilot whales have been affected during the outbreak (Visser, Van Bressem, Barrett, et al. 1993, Fernández et al. 2008, Raga et al. 2008, Van Bressem et al. 2009, Keck et al. 2010).

As in other marine mammal morbillivirus mortality events, the role of pollutants in the striped dolphin Mediterranean epizootic has been a matter of intense debate (De Swart et al. 1995, Kajiwara et al. 2008, Van Bressem et al. 2009, Beineke et al. 2010). Polychlorinated biphenyl (PCB) levels were found to be significantly higher in the dolphins dying during the 1990 epizootic than in the dolphin population sampled before or after the event (Aguilar & Borrell 1994, Borrell et al. 1996). It was hypothesized that PCBs had led to depressed immunocompetence and increased susceptibility to the viral infection (Aguilar & Borrell 1994, Osterhaus et al. 1995). However morbilliviruses are primary pathogenic agents, able to originate die-offs in their susceptible hosts without requiring predisposing factors (Appel et al. 1981, O'Shea 2000). During the 2007 Mediterranean morbillivirus epizootic the OCs levels in the affected dolphins were similar to previous years, supporting the absence of contribution of these substances to the development of this epizootic (Castrillon et al. 2010).

In the years following the 1990 epizootic few striped dolphins occasionally found on the Catalonian Mediterranean coast showed an unusual form of CeMV infection different from the classical systemic presentation, with lesions (non-suppurative meningoencephalitis) and viral antigen limited to the brain. This unusual form resembled subacute sclerosing panencephalitis (SSPE) and old dog encephalitis (ODE), chronic variants of Measles virus (MV) and Canine distemper virus (CDV) infection, respectively (Schneider-Schaulies et al. 1995, Maxie & Youssef 2007), and was considered a chronic infection of CeMV in dolphins (Visa i Esteve 1994, Domingo et al. 1995). More information regarding SSPE and ODE can be found bellow.

As mentioned before the Mediterranean monk seal is the only pinniped inhabiting the Mediterranean Sea. Widely distributed in the Mediterranean and in the eastern Atlantic in the past, nowadays the Mediterranean monk seal is considered by the IUCN red list as "critically endangered". The Mediterranean Sea only counts with around 600 individuals forming a large subpopulation in Greek and Turkey, and with few seals that seem to use the waters of Cyprus and Algeria. Small isolated colonies can be also found between Mauritania and Western Sahara, and around the Madeira islands (Otero & Conigliaro 2012). Large mortality in the population of the Western Sahara monk seal population was reported around May in 1997 (Osterhaus et al. 1997). A morbillivirus designated as Monk seal morbillivirus (MSMV) was isolated (Osterhaus et al. 1997, 1998, Van de Bildt et al. 1999, 2000), but the investigated seals did not show lesions compatible with morbillivirus (Hernández et al. 1998). Algal toxins have been also related with this monk seal mortal event by Hernández et al. (1998), although other investigations showed contradictory results (Osterhaus 1989). So, although a morbillivirus was circulating at the time of the die-off, it is not clear if it was the cause of the monk seals death (Harwood 1998). As the monk seal population is too small and widely dispersed to harbour an endemic virus, the likely source of the morbillivirus found in the seals would be the striped dolphin population, as the MSMV isolated from the monk seals is closely related to DVM (Harwood 1998). This would imply that transmission of morbillivirus between close animal orders can occur (Harwood 1998, Osterhaus et al. 1998). In the late 90's a Mediterranean monk seal was found on the Greek coast showing clinical and histopathological findings reminiscent of a morbillivirus infection. A morbillivirus closely related to another cetacean strain, PMV, was isolated in this case (Osterhaus et al. 1998).

Hooded seals (*Cystophora cristata*), usually inhabiting the central and western North Atlantic Ocean, can migrate as far as the Canary Islands, and have been seen near the Spanish coast at

the Strait of Gibraltar (Bellido et al. 2009). During the 1990 Mediterranean morbillivirus epizootic Osterhaus et al. (1992) found morbillivirus antigen and nucleic acid in several tissues from two hooded seals stranded near Huelva and Tarifa (Spain). These cities are located on the Gulf of Cádiz and near the Strait of Gibraltar, respectively. More information regarding the existence of lesions, the organs affected, or the relation of the detected virus found in these seals with CeMV, is not available.

3. Subacute sclerosing panencephalitis and old dog encephalitis

Mediterranean striped dolphins can show a chronic localized nervous morbillivirus infection in the years following a CeMV epizootic. This unusual presentation of the disease in cetaceans shows similarities to subacute sclerosing panencephalitis due to Measles virus in humans and old dog encephalitis due to Canine distemper virus in dogs.

3.1. Subacute sclerosing panencephalitis

Measles virus (MV), the type species virus of the Morbillivirus genus (King et al. 2012), is a highly contagious agent responsible of measles disease (Schneider-Schaulies et al. 1995). The infection occurs through the oro-nasal route, with primary replication in the upper respiratory tract. Then the virus spreads to the local lymph nodes, where replication leads to viraemia, reaching multiple organs through the blood associated to peripheral blood mononuclear cells, mainly spleen, lymph nodes, skin, lung, gastrointestinal tract and liver. MV has been also identified in cerebral endothelial capillary cells during acute disease. Transient brain infection would probably occur in all individuals, but the development of nervous lesions would vary with the immune status (Schneider-Schaulies et al. 1995, Cosby et al. 2002, Griffin, 2004). The classical disease of acute measles develops after approximately ten days, with fever and cutaneous rash. Complications in measles most commonly consist in pneumonia, diarrhoea and encephalitis (Schneider-Schaulies et al. 1995, Sabella 2010). Acute measles encephalitis, also termed acute measles post-infectious encephalitis (AMPE), occurs during or shortly after acute measles, and is supposed to be an autoimmune disorder induced by the virus (Schneider-Schaulies et al. 2003, Reuter & Schneider-Schaulies 2010). Late neurologic complications can also develop associated to persistent infection and reactivation of MV in the central nervous system, as measles inclusion body encephalitis (MIBE) affecting immunocompromised individuals, and subacute sclerosing panencephalitis (SSPE) affecting fully immunocompetent individuals (Schneider-Schaulies et al. 2003, Yanagi 2006).

SSPE is a rare disease in developed countries, although the incidence still remains high in some poor and developing countries. According to the estimate of the Global Advisory Committee on Vaccine Safety of World Health Organization, the incidence of SSPE is approximately 4–11 cases per 100 000 cases of measles (Campbell et al. 2007, Garg 2008). Most patients affected by SSPE are usually around 5-15 years old, showing most of them a history of acute measles infection at an early age. The virus remains latent after the primary infection and is reactivated 2-10 years later causing a fatal nervous process (Garg 2008). Studies showing that SSPE viral sequences are related with those circulating at the time of the primary infection, and not to those circulating at the time when SSPE signs appear, confirm that SSPE is not due to MV re-infection (Rima & Duprex 2005a). The disease is twice more common in boys than in girls, although primary MV infection shows similar incidence in both sexes (Garg 2002). The usual failure when trying to isolate infectious virus from SSPE brains, the absence of budding viral particles in SSPE infected brain cells, and the strongly reduced expression of proteins despite the presence of ribonucleoprotein particles, suggests that SSPE is caused by replicative defective MV (Baczko et al. 1986, Schneider-Schaulies et al. 1995, 2003, Rima & Duprex 2005a). Viral isolates recovered after much difficulty from SSPE patients can cause demyelinating encephalopathy in experimental animals (Craighead 2000). The study of different parts of the brain in SSPE patients suggests that the virus in the brain is clonal, implying that the virus entered the brain and then gradually spread throughout the nervous tissue (Griffin, 2004). Mutations affecting the Matrix (M) gene, and also the Haemagglutinin (H), the Fusion (F) and the Nucleocapsid (N) genes, have been described in MV strains from SSPE affected patients (Garg 2008). These mutations show no consistent pattern, and different strains from different SSPE patients can show marked differences (Craighead 2000). In SSPE the defective MV viral strains would develop a persistent infection, with failure of the immune system to eliminate the virus. SSPE has been intensively investigated in the last decades, but the mechanisms allowing this persistence are not yet clarified. The synaptic cell-to-cell spread of the viral ribonucleoproteins between nervous cells could contribute to the persistent infection (Schneider-Schaulies et al. 1995, Garg 2008, Reuter & Schneider-Schaulies 2010).

The clinical and histopathological findings associated to SSPE are well established, and define the diagnostic criteria of the process. SSPE patients show progressive subacute mental deterioration with stereotyped generalized myoclonus (periodic myoclonic jerks) and an electroencephalogram with a characteristic periodic pattern. The cerebrospinal fluid (CSF) presents a prominent immunoglobulin elevation, with globulin levels greater than 20% of

total CSF proteins. MV antibody titres are raised in CSF and in serum. Regarding the microscopic findings the affected brain shows a non-suppurative meningoencephalitis involving the grey and white matter. Prominent perivascular cuffing composed of lymphocytes, macrophages and plasma cells are an early lesion. Neuronal degeneration, gliosis and demyelination can be also observed in SSPE patients. The demyelinating lesions would be secondary to the cerebral grey matter and oligondendroglia infection. The parieto-occipital region of the brain is most severely affected, with spreading of the lesions to the anterior portions of the cerebral hemispheres, subcortical structures, brainstem, and spinal cord. The cerebellum is either not affected or presents less damage. Inclusion bodies are seen within both nucleus and cytoplasm of neurons and glial cells. Late in the course of the disease it may be difficult to find typical areas of inflammation and even inclusion bodies, showing the affected tissues marked parenchymal necrosis and gliosis (Drysdale et al. 1976, Craighead 2000, Garg 2002, 2008).

3.2. Old dog encephalitis

Canine distemper virus (CDV) is the agent causing canine distemper in dogs. After infection the virus replicates in lymphoid tissues and, several days after, spreads to various epithelial tissues and the central nervous system (Rudd et al. 2006). Respiratory, digestive, cutaneous and neurological signs can follow. Virus strain, dog age and dog immune status influence the development of nervous lesions. The neurologic signs often occur in the absence of systemic signs, approximately 3 weeks after the infection. In the majority of spontaneous distemper cases, the virus causes demyelinating encephalomyelitis with lesions predominantly located in the cerebellar follia and around the fourth ventricle, optic tracts and spinal cord. In the first acute phase, demyelinating lesions prevail. Afterwards CDV can persist in the brain, becoming the condition chronic. Inflammation of the nervous tissue follows, which can lead to tissue damage (Summers et al. 1995, Vandevelde & Zurbriggen 2005, Caswell & Williams 2007, Beineke et al. 2009, Headley et al. 2009, Vandevelde et al. 2012). CDV is also responsible of other neurological conditions in dogs, as postvaccinal distemper encephalitis and old dog encephalitis (ODE) (Vandevelde et al. 2012).

ODE is an extremely rare chronic consequence of CDV infection, with only six naturally occurring descriptions in the veterinary literature, the last one dating from 2009 (Headley et al. 2009). The disease affects middle-aged dogs frequently with complete vaccination history

that may or may not have history of clinical distemper (Lincoln et al. 1971, Diallo 1990, Axthelm & Krakowka 1998). The affected animals are usually depressed, poorly responsive and have an ataxic gait. Convulsions are rare (Adams et al. 1975, Summers et al. 1995). Typically mental and motor abilities progressively impair, dying after several months. The CSF usually contains high levels of immunoglobulins, and virus neutralizing antibodies can be also found in the serum (Lincoln et al. 1971, Axthelm & Krakowka 1998).

Lesions in ODE are restricted to the brain, mainly the forebrain, affecting both the grey and white matter. The cerebellum and the spinal cord appear unaffected. Affected animals show non-suppurative encephalitis characterized by large perivascular cuffs mainly composed of lymphocytes and plasma cells. Gliosis, fibrillary astrocytosis and neuronal degeneration are also described (Lincoln et al. 1971, Vandevelde et al. 1980, Summers et al. 1995, Axthelm & Krakowka 1998, Maxie & Youssef 2007, Headley et al. 2009). Syncytia can occasionally be observed (Headley et al. 2009). Demyelination can vary depending on the case (Vandevelde et al. 1980). The relevance of this finding in ODE has been interpreted in different ways: for some authors demyelination is not considered a prominent pathologic feature (Lincoln et al. 1971), but for others demyelination in ODE is prominent and diffuse (Adams et al. 1975). Cytoplasmic and nuclear viral inclusions are reported in neurons and astrocytes. Regarding the distribution of the viral antigen, it is mainly located in the grey matter, in neurons and also astrocytes (Lincoln et al. 1971, Summers et al. 1995, Axthelm & Krakowka 1998, Maxie & Youssef 2007, Headley et al. 2009).

Unlike typical forms of canine distemper encephalitis, the virus causing ODE can be isolated only with much difficulty and effort, and the disease is not transmissible by direct inoculation of brain homogenates (Lincoln et al. 1971, Adams et al. 1975, Vandevelde et al. 1980, Summers et al. 1995, Maxie & Youssef 2007). Only once has this condition been experimentally reproduced (Axthelm & Krakowka 1998). The nature and location of the lesions are different between ODE and the classic distemper encephalitis, even when the classic cases are chronic. Research studies suggest that distemper encephalitis does not progress to ODE (Vandevelde et al. 1980).

ODE has been considered similar to SSPE (Adams et al. 1975, Vandevelde et al. 1980, Diallo 1990, Vandevelde & Zurbriggen 1995, Axthelm & Krakowka 1998), and as in SSPE a persistent CDV infection due to a possible replication defect has been hypothesized (Diallo 1990, Summers et al. 1995, Vandevelde & Zurbriggen 1995, Axthelm & Krakowka 1998,

Maxie & Youssef 2007). However although in SSPE the MV virus fails to produce several viral proteins, mainly the membrane associated M protein, the scant studies performed with putative ODE strains and with CDV nervous persistent strains have shown that all major proteins were produced in these cases, including the M protein (Axthelm & Krakowka 1998, Vandevelde & Zurbriggen 2005, Wyss-Fluehmann et al. 2010).

4. Structure of the study, objectives and main findings

This thesis includes four studies: three scientific articles published in international peer-reviewed scientific publications, and one book chapter. Related results obtained after the publication of these works have been also used in the discussion chapter, and have been included in the appendix section. The main objective of the thesis is to compile the pathological and molecular findings we have obtained in the Mediterranean striped dolphins affected by morbillivirus infection during the 2007 Mediterranean morbilliviral epizootic and the following post-epidemic years. The four studies in which these findings have been described are the following:

1. *Epizootic of Dolphin morbillivirus on the Catalonian Mediterranean coast in 2007*. Soto S, González R, Alegre F, González B, Medina P, Raga JA, Marco A, Domingo M (2011) *Veterinary Record* 169(4):101
2. *Systemic herpesvirus and morbillivirus co-infection in a striped dolphin (Stenella coeruleoalba)*. Soto S, González B, Willoughby K, Maley M, Olvera A, Kennedy S, Marco A, Domingo M (2012) *Journal of Comparative Pathology* 146(2-3):269-273
3. *Post-epizootic chronic Dolphin morbillivirus infection in Mediterranean striped dolphins (Stenella coeruleoalba)*. Soto S, Alba A, Ganges Ll, Vidal E, Raga JA, Alegre F, González B, Medina P, Zorrilla I, Martínez J, Marco A, Pérez M, Pérez B, Pérez de Vargas Mesas A, Martínez Valverde R, Domingo M (2011) *Diseases of Aquatic Organisms* 96:187-194
4. *Morbilliviruses in sea mammals.* Soto S, Domingo M (2013) In: Mononegavirales of Veterinary Importance, volumen 1: Pathobiology and Molecular Diagnosis, Munir M (ed.) CABI, United Kingdom, 269-284

The first publication, entitled "*Epizootic of Dolphin morbillivirus on the Catalonian Mediterranean coast in 2007"*, focuses on the pathologic findings observed in the striped dolphins that stranded on the Catalonian Coast in 2007 during the second Morbillivirus epizootic in the Mediterranean Sea. The lesions observed in the studied organs and the detection of morbillivirus antigen in tissues by immunohistochemistry are described. Lesions and immunohistochemical findings were similar to those observed in the first morbillivirus epizootic in 1990 in the Mediterranean Sea. However in the 2007 epizootic the affected dolphins were younger than in the first epizootic (the age of the dolphins was estimated based on the body length measurement of the animals), suggesting that older dolphins may have been protected from the infection due to a previous exposure to the virus in the 1990 epizootic. In addition in this paper evidences are provided indicating that CeMV has not circulated on the Catalonian coast in the years between the 1990 and the 2007 morbillivirus epizootics in the Mediterranean Sea.

One of the striped dolphins studied in the 2007 morbillivirus epizootic showed, in addition to the morbillivirus infection, systemic lesions compatible with herpesvirus infection. The herpesvirus was ultrastructurally detected, and a pan-herpesvirus nested PCR confirmed an alphaherpesvirus co-infection. This striped dolphin is, to the best of our knowledge, the first cetacean co-infected with both viruses showing lesions attributable to both of them. It was described with detail in our second publication entitled "*Systemic herpesvirus and morbillivirus co-infection in a striped dolphin (Stenella coeruleoalba)"*.

In the years following the 1990 morbillivirus epizootic several striped dolphins showed a chronic morbillivirus infection that, in contrast to the systemic form observed during the epizootic, was characterized by lesions and viral antigen localized only in the brain (Domingo et al. 1995). In consequence our group dedicated special effort in the study of dolphins appearing dead on the Mediterranean coasts during the years following the 2007 morbillivirus epizootic. Histological, immunohistochemical and molecular studies for the diagnosis of morbillivirus infection were performed in 118 dolphins found stranded along the Mediterranean coast of Catalonia, Valencia Region and Andalusia from January 2008 to December 2010. During this period 27.1% of the studied striped dolphins showed the nervous chronic form of CeMV infection, characterized by non-suppurative encephalitis with mononuclear perivascular cuffs and spongiotic changes, usually more prominent in the forebrain. The cerebellum was usually unaffected. Morbillivirus viral antigen and viral RNA were detected only in the brain. These data indicated that this form of morbillivirus infection

is the most relevant infectious cause of striped dolphin stranding in the western Mediterranean Sea in the years following a morbillivirus epizootic. In addition the statistical analysis of the recorded epidemiological data showed that the affected dolphins were longer (thus older) than the non-affected dolphins. These results were reported in our third publication, entitled "*Post-epizootic chronic Dolphin morbillivirus infection in Mediterranean striped dolphins (Stenella coeruleoalba)".*

These three articles mentioned above were published in international peer-reviewed journals in 2011 and 2012. Few months after the third article was published we received an invitation from the editor of the book "Mononegavirales of Veterinary Importance, volumen 1: Pathobiology and Molecular Diagnosis" to write the chapter focused in morbilliviruses affecting marine mammals: "Morbilliviruses in sea mammals". This book chapter, also enclosed in this thesis, summarizes the main epidemiological, clinical, pathological and diagnostic available data regarding morbillivirus infection in cetaceans and pinnipeds.

In addition to our published work we have included in the appendix section of the thesis data obtained in the course of the morbillivirus survey in the Mediterranean Sea from January 2011 to April 2014. During this time, our group has evaluated tissue samples from dolphins stranded in Catalonia, Valencia Region and Andalusia. Microscopic analysis of lesions, and immunhistochemistry and RT-PCR techniques for the detection of morbillivirus, have been performed in order to detect new cases in the Mediterranean suffering the morbillivirus infection. Five animals out of 68 analysed striped dolphins have shown the chronic form of morbillivirus disease, with lesions and viral antigen/viral nucleic acid restricted to the brain. These five dolphins stranded along 2011 and 2012. From 2013, no new morbillivirus chronic case has been diagnosed in our service. Our group hypothesized in our third article the absence of new chronic cases after 2012, based on the temporal stranding pattern of chronic cases after the 1990 Mediterranean morbillivirus epizootic, and the apparent decrease trending of chronic cases from 2008 to 2010. However more time is needed to confirm if the prediction was correct. In addition two juvenile striped dolphins out of these 68 have been found showing the systemic form of morbillivirus infection. Both dolphins stranded in the area of the Strait of Gibraltar, where the Mediterranean Sea and the Atlantic Ocean meet. One dolphin was found stranded in December 2011, and the other one in August 2012. They were diagnosed as single systemic cases.

PUBLICATIONS

1. Epizootic of Dolphin morbillivirus on the Catalonian Mediterranean coast in 2007

Soto S, González R, Alegre F, González B, Medina P, Raga JA, Marco A, Domingo M (2011) *Veterinary Record* 169(4):101

Doi: 10.1136/vr.d1686

2. Systemic herpesvirus and morbillivirus co-infection in a striped dolphin (*Stenella coeruleoalba*)

Soto S, González B, Willoughby K, Maley M, Olvera A, Kennedy S, Marco A, Domingo M (2012) *Journal of Comparative Pathology* 146(2-3):269-273

Doi: 10.1016/j.jcpa.2011.04.002

3. Post-epizootic chronic Dolphin morbillivirus infection in Mediterranean striped dolphins (*Stenella coeruleoalba*)

Soto S, Alba A, Ganges Ll, Vidal E, Raga JA, Alegre F, González B, Medina P, Zorrilla I, Martínez J, Marco A, Pérez M, Pérez B, Pérez de Vargas Mesas A, Martínez Valverde R, Domingo M (2011) *Diseases of Aquatic Organisms*, 96:187-194

Doi: 10.3354/dao02387

4. Morbilliviruses in sea mammals

Soto S, Domingo M (2013) In: Mononegaviruses of Veterinary Importance, volumen 1: Pathobiology and Molecular Diagnosis. Munir M (ed.), CABI, United Kingdom, 269-284

Doi: 10.1079/9781780641799.0269

GLOBAL DISCUSSION

The morbillivirus epizootic affecting the western Mediterranean Sea in 2006 and 2007 supposed the re-emergence of the disease in these waters after the first outbreak, that occurred in 1990 (Domingo et al. 1990, Raga et al. 2008). Factors leading to the repetition of mass-mortalities in previously affected areas are usually poorly understood, but a clearance of the infection in the affected populations with a build-up of susceptible individuals during these years would probably occur. The morbillivirus serological data available from western Mediterranean dolphins from 1997 to 1999 support this hypothesis, indicating a decrease in the prevalence of mobillivirus seropositive dolphins (Van Bressem et al. 2001). In our first publication enclosed in this thesis ("*Epizootic of Dolphin morbillivirus on the Catalonian Mediterranean coast in 2007*") we performed a retrospective morbillivirus study using immunohistochemistry in 58 cetaceans stranded on the Catalonian coast from 1995 to 2005, obtaining negative results in all cases. These findings would also support the hypothesis of absence of circulation of Cetacean morbillivirus (CeMV) in the inter-epizootic period in western Mediterranean waters. However it cannot be fully excluded that CeMV would have circulated in the western Mediterranean Sea without evidence of disease for the chosen diagnostic tools, and without evidence of increased mortality.

During the second epizootic the dolphin population suffered the disease in 2007, with the striped dolphin (*Stenella coeruleoalba*) again as main protagonist, as occurred in 1990. However in the 2007 outbreak not only this species but also bottlenose dolphins (*Tursiops truncatus*) and long finned pilot whales (*Globicephala melas*) were found stranded due to the viral infection (Fernández et al. 2008, Keck et al. 2010). The fact that the striped dolphin is the most abundant cetacean in the Mediterranean Sea (Otero & Conigliaro 2012) could explain the relatively higher impact of the disease in this species, as serological studies performed from 1997 to 1999 showed that other Mediterranean cetaceans, as Risso's dolphins (*Grampus griseus*) and bottlenose dolphins, can also be infected by CeMV (Van Bressem et al. 2001). Nonetheless an increased susceptibility of striped dolphins to the disease cannot be excluded.

The 2007 epizootic was milder in severity and extension (Castrillon et al. 2010, Di Guardo et al. 2010) than the 1990 epizootic. This lower impact is probably related to the residual protection in the dolphins against the virus after the first outbreak (Van Bressem et al. 2001). The measurement of the body length in stranded dolphins has shown that those affected during the 2007 epizootic on the Valencia Region coast and the French coast were shorter

than those affected during the 1990 epizootic (Raga et al. 2008, Keck et al. 2010). We also measured the body length of the dolphins stranding on the Catalonian coast during the 2007 epizootic, confirming the shorter length of these dolphins. As the dolphin length correlates with the sexual maturity, and the body length has been usually used to estimate dolphin's age, the dolphins found stranded during the 2007 morbillivirus epizootic would be younger (mainly juveniles) than the dolphins found stranded during the 1990 epizootic (mainly adults) (Raga et al. 2008, Keck et al. 2010). These findings would support the hypothesis of residual immunity protecting the dolphin population during the 2007 morbillivirus outbreak. However other factors as inferior number of social contacts and genetic selection of less susceptible individuals originating from survivals of the first epizootic could also be postulated (Müller et al. 2004). CeMV does not seem to have preference for male or female dolphins. Both sexes were similarly affected in the 1990 epizootic (Calzada et al. 1994, Visa i Esteve 1994) and the same finding has been observed in the dolphins stranded in France and in Catalonia in the 2007 epizootic (Keck et al. 2010, Soto et al. 2011).

The lesions recorded in the dolphins affected during the 2007 epizootic by other authors (Raga et al. 2008, Keck et al. 2010) and by us are similar to those described in other dolphin morbillivirus epizootics, and similar to those described in pinnipeds and terrestrial mammals affected by morbilliviruses. The main lesions are three: bronchiolo-interstitial pneumonia, lymphoid necrosis/depletion and non-suppurative meningoencephalitis, with associated syncytial cells and nuclear/cytoplasmic inclusions (Domingo et al. 1992, Duignan et al. 1992, Lipscomb et al. 1994, Kennedy 1998, Birkun et al. 1999, Müller et al. 2004, Caswell & Williams 2007). The damage caused in the lymphoid tissues generates immunosuppression in the affected animals, usually followed by opportunistic infections (Domingo et al. 1992, Kennedy 1998). As described in the dolphins affected during the 1990 Mediterranean epizootic, lesions compatible with secondary aspergillosis and toxoplasmosis were also observed in the 2007 epizootic affecting the Catalonian coast. In addition one of the dolphins found in Catalonia presented herpesvirus infection concomitant to the morbillivirus infection, to our knowledge the first time that both infectious agents are found causing disease at the same time in a cetacean.

The dolphin showing fungal lesions during the 2007 morbillivirus epizootic in Catalonia showed the same necro-haemorrhagic lesional pattern in brain and lungs as the striped dolphins infected by *Aspergillus fumigatus* in the 1990 Mediterranean morbillivirus epidemic,

and as the western Atlantic bottlenose dolphins infected by *Aspergillus spp*. during the morbillivirus epizootic in the United States Atlantic coast in 1987-1988 (Domingo et al. 1992, Lipscomb et al. 1994, Visa i Esteve 1994, Schulman et al. 1997). These findings would suggest that *Aspergillus* spp. usually causes this pattern of lesions in dolphins when a morbillivirus infection is active. Pulmonary aspergillosis is the most common mycotic infection in cetaceans, and the majority of the descriptions in free-ranging cetaceans correspond to dolphins implicated in morbillivirus epizootics (Delaney et al. 2013, Stephens et al. 2014). In the Catalonian coast these fungal lesions have only been observed in cetaceans affected during a morbillivirus outbreak. Pneumonic lesions caused by *Aspergillus* spp. were also described in the pilot whales affected by the morbillivirus infection in 2006 and 2007 in the Mediterranean Sea (Fernández et al. 2008).

The immunosuppressive effect of morbillivirus infection on the dolphin populations is also reflected by the frequent synergistic appearance of toxoplasmosis with the viral epizootic, increasing the severity of the viral disease (Van Bressem et al. 2009). Similar to the 1990 morbillivirus Mediterranean epizootic (Domingo et al. 1992), during the 2007 epizootic we observed two striped dolphins showing lesions caused by *Toxoplasma gondii*. As occurred in the 1990 epizootic half of the dolphins affected by toxoplasmosis in the 2007 epizootic were positive for the morbillivirus immunohistochemistry while half of them were negative. These negative cases were considered individuals that would have overcome the morbillivirus infection but died due to the *T. gondii* infection. How *T. gondii* infection gets to affect offshore species as the striped dolphin is not clear; contaminated ship run-off waters have been suggested to be linked to this infection (Van Bressem et al. 2009). On the Catalonian coast all dolphins studied since 1990 until 2011 have shown *T. gondii* associated lesions only in the frame of the morbillivirus epizootics, in spite of the 11% *T. gondii* seroprevalence in dolphins described by Cabezón and others (Cabezón et al. 2004) in the Spanish Mediterranean waters. However in October 2011 a striped dolphin was found on the southern Spanish Mediterranean coast (Andalusia) suffering encephalitis associated to *T. gondii* infection. No morbillivirus epizootic was active in the Mediterranean Sea in that moment, but less than two months later another striped dolphin was found stranded on the same Andalusian region affected by morbillivirus infection. This dolphin was a single case suffering the systemic form of CeMV. Whether these cases are related is unknown. Both striped dolphins were found stranded after the time our articles were published; they are described in the appendix chapter of this thesis.

An intriguing *T. gondii* infection has been described affecting striped dolphins found stranded in the north-western Italian Mediterranean Sea in the second half of 2007 (Di Guardo et al. 2010). Considering the epidemiological and serological results these dolphins would seem to have died due to morbillivirus infection, as the mortality occurred shortly after the beginning of the 2007 Mediterranean morbillivirus epidemic and half of the studied dolphins showed seropositive results for morbillivirus (Garibaldi et al. 2008). However the histopathological, immunohistochemical and RT-PCR studies performed afterwards in nine striped dolphins, one stranded on northwestern Sardinia in June 2007, and eight stranded on the Ligurian coast from August to December 2007, did not support this assumption. Only the dolphin found in Sardinia showed brain demyelination and positive morbillivirus immunostaining in brain cells. But classical dolphin morbillivirus lesions and detection of morbillivirus by immunohistochemistry and RT-PCR in other target tissues have not been observed in this mortality event (Appino et al. 2008, Di Guardo et al. 2010, 2011). By the contrary, the half of the studied dolphins that did not show detectable antibodies against morbillivirus were seropositive for *T. gondii* and showed a non suppurative meningoencephalitis with *T. gondii* antigen in two brain samples. Whether these dolphins showing toxoplasmosis correspond to individuals that had overcome a pre-existent morbillivirus infection, or to dolphins that had not suffered morbillivirus infection, it is not known. The negative results for morbillivirus detection would be more in accordance with the second explanation (Di Guardo et al. 2010, 2013).

One of the striped dolphins stranded on the Catalonian coast in the 2007 epizootic showed co-infection by morbillivirus and by an Alphaherpesvirus, with lesions attributable to both infectious agents. Coinfection of striped dolphins with theses viruses has been described in other occasions in the Mediterranean Sea and the Northeast Atlantic Ocean, but without evident lesions caused by the herpesvirus (Bellière et al. 2010, Sierra, Sánchez et al. 2014). The striped dolphin found in Catalonia was described in the paper "*Systemic herpesvirus and morbillivirus co-infection in a striped dolphin (Stenella coeruleoalba)"* enclosed in this thesis. Systemic herpesvirosis has been rarely described in cetaceans (Kennedy, Lindstedt et al. 1992, Blanchard et al. 2001, Arbelo et al. 2010), and the striped dolphin stranded in Catalonia was, to the best of our knowledge, the first cetacean described in the Mediterranean Sea showing systemic lesions attributable to an active herpesvirus infection. However several herpesvirus sequences have been detected in other striped dolphins stranded in Mediterranean waters during the 2007 epizootic, one of them showing high homology to the herpesvirus

found in the case from Catalonia (Bellière et al. 2010). These results highlight the importance of herpesvirus infection in the striped dolphin population in the Mediterranean Sea. In addition the herpesvirus found in the dolphin from Catalonia shows close proximity with two herpesvirus strains found in cetaceans stranded in the Canary Islands, one Cuvier´s beaked whale (*Ziphius cavirostris*) found in 2005 also affected by systemic herpesvirosis (Arbelo et al. 2010), and one striped dolphin found in 2007 affected by herpesviral meningoencephaltitis (Sierra, Sánchez et al. 20014). These findings point out again the apparent dynamism of infectious agents through the Strait of Gibraltar (Sierra, Sánchez et al. 20014). The pathological findings observed in the striped dolphin described in our article would suggest that the herpesvirus infection was preceded by the morbillivirus infection, but in other cetaceans affected by systemic herpesvirosis predisposing factors or primary infections have not been described. Further studies would be required in order to understand the role of herpesvirus causing systemic disease in cetaceans.

Until this point of the chapter we have discussed about the morbillivirus disease affecting striped dolphins during the 2007 Mediterranean morbillivirus epizootic, and its similarities and differences with the 1990 morbillivirus Mediterranean epizootic. From here on we will focus our discussion in a different presentation of the morbillivirus disease in dolphins named the chronic form. This form of the disease was observed and described by our group affecting striped dolphins stranding on Catalonia in the years following the 1990 Mediterranean morbillivirus epizootic, once the epidemic had already subsided the Catalonian coast (Domingo et al. 1995). The last dolphin affected by the 1990 epizootic in Catalonia was found the 31st October 1990. From January 1991 to May 1994 five striped dolphins out of 27 dolphin strandings (18.5%) where found in Catalonian waters affected by a morbillivirus infection characterized by lesions and viral antigen restricted to the brain. The presentation of the infection in these five dolphins was considered different to the presentation of the infection during the morbillivirus epizootic, as: (1) the five dolphins were found along nearly 3.5 years in a sporadic pattern, contrary to the 1990 Mediterranean morbillivirus epizootic, where hundreds of dolphins died in few months because of the infection; (2) the five dolphins showed lesions and viral protein only in the brain, whereas in the 1990 Mediterranean morbillivirus epizootic lesions and viral antigen were found in multiple organs, mainly in lungs, lymphoid organs and brain. The presentation of the morbillivirus infection in these five dolphins was then considered a new form, referred as chronic (Domingo et al. 1995). The *systemic form* of the morbillivirus infection in dolphins would be similar to the classic

systemic infection caused by morbillivirus in other animal species. After the infection, most probably via the oro-nasal route, the virus would replicate in regional lymphoid tissues, as the laryngeal gland (Smith et al. 1999, Beineke et al. 2010). From there it would spread and replicate systemically being the lung, the lymphoid tissues and the brain the main target organs of the virus, and so the organs showing the main lesions and showing virus antigen with more frequency (Kennedy et al. 1989, Domingo et al. 1992, Visa i Esteve 1994). The *chronic form* of the morbillivirus infection in dolphins would probably evolve after a primary infection during an epizootic phase. The virus would persistently infect the dolphin brain tissue, reactivating several months or years later and causing a fatal disease restricted to the brain, with absence of lesions and virus in other tissues (Visa i Esteve 1994, Domingo et al. 1995).

The re-emergence of the morbillivirus epizootic in the Mediterranean Sea in 2007 was an opportunity for our group to follow up the appearance of new chronic cases during the following years, with the additional advantage of having this time a wider sampling covering most of the Spanish Mediterranean coast: Catalonia, Valencia Region and Andalusia. The results of this investigation have been described in the publication *"Post-epizootic chronic Dolphin Morbillivirus infection in Mediterranean striped dolphins (Stenella coeruleoalba)"*, included in this thesis. The last dolphin affected during the 2007 epizootic in Catalonia was found the 23th December 2007; afterwards the epizootic was considered to be over on the Catalonian coast. From January 2008 to December 2010, 32 out of 118 striped dolphins (27.1%) were found showing a CeMV infection with the characteristics of the described chronic form: lesions and viral antigen located only in the brain, and usually sparing the cerebellum. To assess the restriction of the virus in the brain tissue, frozen samples from the organs target for the virus during the systemic infection (brain, lung and lymph nodes) were collected from 17 out of the 118 dolphins and used to perform a RT-PCR study to detect DMV. This technique proved the existence of DMV nucleic acid only in dolphins showing morbillivirus lesions and antigen in the brain, and it also proved the location of the nucleic acid only in the brain of these dolphins. All together these findings showed that during the years following the 2007 Mediterranean morbillivirus epizootic the chronic morbillivirus form of infection has affected the striped dolphins in the Spanish Mediterranean coast as ocurred after the 1990 epizootic, and confirms the relevance of this form of the disease, causing high mortality in the striped dolphin population along the Spanish Mediterranean

coast. In the 2007 epizootic the effect of the chronic form seems to have even surpassed the effect of the epizootic itself.

The finding of dolphins affected by the chronic form of morbillivirus infection in Valencia Region and Andalusia in addition to Catalonia, showing similar percentages in the three regions (28.6% in Valencia Region, 27.4% in Andalusia and 25% in Catalonia) showed that this was an extended event in the Spanish Mediterranean coast, not regionally located. The temporal distribution of the dolphins affected by the chronic form after the 2007 epizootic presented an apparent reduction trend from 2008 to 2010. In the publication mentioned above, considering the absence of new chronic cases nearly 3.5 years after the 1990 epizootic, we hypothesized that the declining trend would go further until not finding new cases on the Spanish coast after 2012. In order to assess this presumption, we have reviewed the striped dolphins evaluated by our group stranded in Catalonia, Valencia Region and Andalusia from January 2011 to April 2014. The detailed information about these strandings can be found in the appendix section. Three out of 25 (12%) striped dolphins, and two out of 24 (8.3%) striped dolphins, have shown the diagnostic features of the chronic morbillivirus infection form in 2011 and 2012, respectively. In 2013 and 2014 no additional case out of the 19 analysed striped dolphins has been found suffering the chronic form of morbillivirus infection. More time should pass, but with the available information we have today the prediction we made seems close to reality.

The statistical analysis performed after the 2007 morbillivirus Mediterranean epizootic indicated that the dolphins affected by the chronic form of the morbillivirus infection were longer, by assumption older, than the dolphins not affected (median body lengths were 196.5 cm versus 160.5 cm, respectively). One explanation for this finding could be that a previous exposure to the virus during the 1990 epizootic would facilitate the development of the chronic form when confronted to the virus in a second occasion. However we do not have the possibility to confirm or refute this hypothesis.

Dolphins showing features similar to the morbillivirus chronic form described on the Spanish Mediterranean coast have been reported in other parts of the Mediterranean Sea. Tsur et al. (1997) described a bottlenose dolphin stranded on Israel in August 1994 showing a morbillirivus infection with non-suppurative encephalitis as the only relevant lesion, and with viral antigen restricted to the brain. Considering that the 1990 Mediterranean epidemic was

reported to reach the nearby Turkish coast during the summer 1992 (Aguilar & Raga 1993, Visser, Van Bressem, Barrett, et al. 1993, Visa i Esteve 1994), this bottlenose dolphin could represent a post-epidemic chronic CeMV case. Bottlenose dolphins were not found affected during the 1990 epizootic (Aguilar & Raga 1993, Tsur et al. 1997), but this species have been shown to suffer the morbillivirus infection in the Mediterranean Sea during the 2007 epizootic, in much less proportion than the striped dolphin (Keck et al. 2010). The low number of bottlenose dolphins in the Mediterranean Sea could be one of the factors involved (Otero & Conigliaro 2012). Other reported dolphins that would meet the criteria for the chronic form of morbillivirus infection have been described in Italy, in the Thyrrenian Sea. One adult bottlenose dolphin stranded in these waters in June 2011 showed non-suppurative encephalitis, with morbillivirus nucleic acid only in the brain (Di Guardo et al. 2013). In the same period Di Guardo et al. (2013) also described in this sea two adult striped dolphins that, although not showing nervous lesions, presented positive morbillivirus RT-PCR in the brain, with negative results in other tissues. One of these dolphins also showed positive morbillivirus immunolabelling restricted to the brain tissue. These cases would be, in opinion of the authors, remarkably similar to the morbillivirus chronic form described in Spain. Considering when these dolphins were found on the Italian coast they could fit as post-epidemic cases after the 2007 Mediterranean morbillivirus epizootic. However, as mentioned before, to the best of our knowledge the available data do not allow to confirm the extension of the 2007 morbillivirus epizootic to the Italian coast.

Why the chronic form has not been described in other Mediterranean regions affected by the 1990 and the 2007 morbillivirus epizootics during the post-epidemic years, why when apparently described in some areas it does not show the same importance as in the Spanish Mediterranean coast, and why it has not been described in other dolphin morbillivirus epizootics occurred in other points in the planet are constant questions we have asked ourselves along these research years. And we still have no answer for them. Perhaps the brain is not routinely sampled and studied in all the stranded dolphins, as seems to have occurred in bottlenose dolphin morbillivirus epizootics in the Atlantic coast of the United States (Lipscomb et al. 1994, 1996). Perhaps the diagnostic protocol for the dolphins stranded out of the epidemic years does not always include the systematic performance of immunohistochemical/RT-PCR techniques for the diagnosis of morbillivirus. Perhaps findings similar to what we consider the chronic form of the disease have been observed in

other coasts, but they have not been considered relevant enough to be published. Or perhaps dolphins affected by the chronic form of the morbillivirus infection have just not been found.

Out of the Mediterranean Sea, several striped dolphins showing non-suppurative meningoencephalitis with detection of morbillivirus limited to brain tissue have been observed in the Canary Islands, in the Northeast Atlantic Ocean (Sierra, Sánchez et al. 2014). The date of these strandings is not indicated, but it would be difficult to find a link with previous systemic morbillivirus events anyway, as this disease is unusual in these waters (Arbelo et al. 2013, Sierra, Zucca et al. 2014, Sierra, Sánchez et al. 2014). A Pacific one-sided dolphin (*Lagenorhynchus obliquidens*) found in Japan, and two white-beaked dolphins (*Lagenorhynchus albirostris*) found in Germany and in Netherlands, have been also described suffering non-suppurative encephalitis as main lesion, with presence of morbillivirus antigen only in the brain (confirmed by RT-PCR for the white-beaked dolphins) (Uchida et al. 1999, Wohlsein, Müller et al. 2007, Van Elk et al. 2014). Uchida et al. and Van Elk et al. consider that the cases they describe would show similarities with the Mediterranean dolphins affected by the morbillivirus chronic form. However the Pacific one-sided dolphin and the white-beaked dolphin are single cases, with no other report of morbillivirus infection before they were found.

The chronic form of morbillivirus infection affecting the striped dolphins was already proposed by our group to resemble subacute sclerosing panencephalitis (SSPE) and old dog encephalitis (ODE) after the 1990 epizootic (Visa i Esteve 1994, Domingo et al. 1995). SSPE and ODE are rare neurologic diseases affecting humans and dogs, respectively, that share similar features (Adams et al. 1975, Vandevelde et al. 1980, Diallo 1990, Vandevelde & Zurbriggen 1995, Axthelm & Krakowka 1998). Both are rare and fatal neurologic syndromes associated to morbillivirus persistent infection in the central nervous system: measles virus (MV) in SSPE cases and distemper virus (DV) in ODE cases. Microscopically, both syndromes are characterised by a non-suppurative meningoencephalitis affecting the grey and white matter mainly in the forebrain and the brainstem, with usual sparing of the cerebellum. Formation of perivascular mononuclear cuffs is a main finding, and viral inclusions can be found in neurons or glial cells. Virus can be recovered from brain tissue of affected individuals and dogs only with much difficulty, although viral antigens and nucleic acids can be easily detected in this tissue. The direct transmission of these diseases is also problematic (Drysdale et al. 1976, Baczko et al. 1986, Schneider-Schaulies et al. 1995, Summers et al.

1995, Axthelm & Krakowka 1998, Craighead 2000, Garg 2002, Schneider-Schaulies et al. 2003, Maxie & Youssef 2007, Garg 2008, Vandevelde et al. 2012). Genetic studies in SSPE specimens have shown that the viral strains are replicative defective MV, showing mutations mainly affecting the matrix (M) protein and other envelope proteins (Schneider-Schaulies et al. 2003). ODE has been also proposed to be caused by defective CDV strains, but the limited studies performed have not yet confirmed it (Diallo 1990, Summers et al. 1995, Vandevelde & Zurbriggen 1995, Axthelm & Krakowka 1998, Vandevelde & Zurbriggen 2005, Maxie & Youssef 2007, Wyss-Fluehmann et al. 2010).

Although SSPE has been extensively studied, the mechanism allowing the virus to persist in the patient during the period between the infection and the development of the clinical signs are not clear. It is still not known whether the virus reaches the brain at the time of the primary infection and persists there, or whether the infection is latent in non-nervous tissues and reaches the brain shortly before the beginning of the symptoms (Griffin 2004, Rima & Duprex 2005a, b). Several authors have reported the finding of MV in blood leukocytes and several non-nervous tissues in SSPE patients using immunohistochemical and in situ hybridisation techniques (Fournier et al. 1985, 1988, Brown et al. 1989). However these studies have been reviewed by Rima and Duprex in 2005 and were considered inconsistent and differing from other published and non-published works that more consistently had shown the absence of MV in other tissues than brain in SSPE (Schneider-Schaulies et al. 1991, Rima & Duprex 2005b). Posterior investigations related to this subject presented, again, conflicting results: Kühne Simmonds et al. (2006) found MV nucleic acid in thymus and appendix tissues in one (out of two) SSPE patient, but the authors agreed that the low amount detected and the possible contamination during the tissue sampling would support the restricted location of the virus in nervous tissues. However Fernández-Muñoz et al. (2011) could find MV nucleic acid in several lymph node samples in two (out of two) SSPE patients. The authors refer to have sampled the tissues themselves in a clean-way, and that the viral sequences obtained from the lymph nodes showed different mutations than the viral sequences obtained from the brain. The scant work reporting information about the location of virus in ODE cases suggests the restricted location of the virus in the brain tissue (Headley et al. 2009).

Considering the available information about SSPE and ODE, and contrasting it with what we know about the chronic form of morbillivirus infection in striped dolphins, there are several

points that would suggest the resemblance of this form of the disease to SSPE and ODE. Dolphin chronic cases have been only diagnosed sporadically after a morbillivirus epizootic has occurred, and only during a period from few weeks to several years once the epizootic is over. This could suggest that the process only develops time after the dolphins have been in contact with the virus and that the dolphins do not probably excrete virus. The lesions observed in the brain of the affected dolphins are similar in nature and in distribution to the lesions described in SSPE and ODE. In addition morbillivirus antigen and nucleic acid have been only detected in brain tissues in the dolphins affected by the chronic form, suggesting the possibility of restricted location of the virus in the brain. However although we consider these similarities enough to hypothesize the resemblance of the dolphin morbillivirus chronic form to SSPE and ODE, further research should be done to really prove it. It should be checked if the chronic form in dolphins is due to morbillivirus strains acquired during the epizootic phase and not during a posterior re-infection; if these strains are defective; and if they establish a latent infection in the dolphins. The amount of resources that these investigations would imply is beyond our reach. In addition working with wildlife marine species as dolphins makes the work even harder, as the in-vivo experiments are quite impossible and the availability of samples is quite restricted. Our group has already initiated the sequencing and comparison of viral strains obtained from dolphins suffering the morbillivirus chronic and systemic forms. But the lack of funding for this investigation is complicating the result accomplishment.

As an alternative option it could be hypothesized that the chronic morbillivirus form affecting the striped dolphins would be just the chronic evolution of the encephalitis acquired during the epidemic phase. In dogs the classical demyelinating encephalitis caused by CDV, different from the ODE encephalitis (Vandevelde et al. 1980), can follow this course. The initial demyelinating lesions occur approximately 3 weeks after the dog gets infected, sometimes without any other signs. The evolution of the process would depend on the immune state of the animal, and would vary from rapid resolution associated to recovery or death, to an intermediate state developing slow, chronic, and even relapsing encephalitis. In this chronic phase inflammation in the brain tissue becomes a relevant lesion, in addition to the loss of myelin. CDV would persist in the brain, out of the lesions, and the encephalitis would progress in time as the immune system of the dog keeps lagging behind viral replication (Vandevelde & Zurbriggen 1995, 2005, Sips et al. 2007). There is no molecular evidence for defective viruses, while viral cultivation and transmission of the disease are usually

accomplished in this form of CDV (Vandevelde et al. 1980, Vandevelde & Zurbriggen 2005). The presence of the virus in non-nervous tissues in CDV classical chronic cases has been rarely investigated. We have found one publication describing detection of CDV antigen in the brain in three out of three dogs suffering distemper chronic demyelination, with negative results in the respiratory tract and the spleen. However one of these dogs showed CDV nucleic acid in serum, whole blood and cerebrospinal fluid (Frisk et al. 1999). The dolphins suffering the chronic form of morbillivirus infection could be suffering a process similar to CDV classical chronic cases. The fact that in the 2007 Mediterranean morbillivirus epidemic several dolphins affected by this form were found only few weeks after the epidemic was subsided would better agree with this hypothesis than with the SSPE one, as in SSPE the clinical signs develop 2-10 years after the infection (Garg 2008). However considering that dolphins depend totally on themselves, and that nervous impairment would probably make them strand and consequently die, it would be quite unlikely that a wild-living dolphin could stand a brain chronic inflammation for several years. In addition no dolphin affected by the morbillivirus chronic form has been found during the several months that both Mediterranean epidemics have lasted. Anyway, as mentioned before, the dolphin chronic form should be deeply investigated, beyond our first approach, in order to clarify the pathogenesis and the viral persistence mechanisms in this disease.

In closing this discussion section I would like to include some comments regarding the publications of Rubio-Guerri *et al.* in 2013 (Rubio-Guerri, Melero, Esperón, et al. 2013, Rubio-Guerri, Melero, Rivera-Arroyo, et al. 2013). In these papers the authors suggest that a third morbillivirus epizootic could have affected the Mediterranean dolphins from March to April 2011, based on the pathological, immunohistochemical and RT-PCR analysis performed in nine striped dolphins stranded during a dolphin mortality occurred in Valencia Region. In our opinion the microscopic findings described in these dolphins are not the usual triad affecting dolphins in a morbillivirus epizootic (Domingo et al. 1992, Duignan et al. 1992). Non-suppurative encephalitis has been observed in three dolphins, with positive morbillivirus immunostaining in brain tissue only in one case. However lymphoid necrosis/depletion of lymphoid tissues has not been observed in any of the studied dolphins, and viral antigen could not be detected in the studied lymph nodes. Broncho-interstitial pneumonia has been described in two dolphins, with associated syncytial cells in one case, but viral proteins could not be detected in the pulmonary tissues in any case. The authors based the diagnosis of systemic morbillivirus infection, in addition to the described lesions, in

the detection of morbillivirus nucleic acid in the tissues of the dolphins. A new Universal Probe Library RT-PCR (UPL RT-PCR) targeting a sequence within the DMV fusion (F) gene, proved to be more sensitive, more specific, and able to differentiate the three CeMV strains, was described in these publications. The analysis of brain and non-nervous tissue samples using the new UPL RT-PCR detected morbillivirus nucleic acid in seven out of the nine striped dolphins: two out of the three dolphins suffering encephalitis showed positive UPL RT-PCR in the brain and other non-nervous tissues; positive results in several tissues were also observed in other dolphins with no lesional or immunohistochemical evidence of morbillivirus disease, but the lung samples with broncho-interstitial pneumonia were negative using this technique. Considering these results, in addition to the absence of increased dolphin mortality and of dolphins affected by the systemic morbillivirus form in the nearby coasts of Andalusia and Catalonia in that period of time, the peak of dolphin mortality detected in Valencia Region from March to April 2011 did not match the features of other dolphin morbillivirus epizootics. In our opinion the authors have proved the existence of dolphins showing morbillivirus infection in different organs, but not systemic disease. The two dolphins showing brain inflammation with nucleic acid detection in brain tissues could be dolphins affected by the chronic form of the disease, although the detection of morbillivirus nucleic acid in non-nervous tissues in these dolphins would be in conflict with the restricted brain location of the virus described by our group in these cases. The increased sensitivity of this UPL RT-PCR compared with conventional RT-PCRs would be one explanation. Whether the virus in these non-nervous tissues would be located in the tissue itself, or in the nervous axons branching off in those tissues, would be an interesting aspect to investigate and discuss, one more in the long list of questions to figure out regarding morbillivirus infections in cetaceans.

CONCLUSIONS

1. The 2007 Mediterranean morbillivirus epizootic affected striped dolphins (*Stenella coeruleoalba*) in the Catalonian coast. Similar to other regions, in Catalonian waters this epizootic was less severe (caused less mortality) than the previous Mediterranean morbillivirus epizootic in 1990.

2. Secondary infections caused relevant lesions in the striped dolphins stranded in Catalonia during the 2007 epizootic, as in the 1990 epizootic. However, in addition to *Toxoplasma gondii* and *Aspergillus* spp., alphaherpesvirus should be also kept in mind as an opportunistic agent, as these viral strains have been detected in several striped dolphins affected during the 2007 epizootic, causing concomitant systemic disease in one of these dolphins.

3. The length of the striped dolphins affected by the 2007 morbillivirus epizootic in Catalonia was shorter than in the 1990 epizootic, as has been observed in other Mediterranean regions affected by the 2007 epizootic. This would suggest that older dolphins might have been protected from CeMV by previous contact during the 1990 epizootic.

4. The immunohistochemical studies performed in cetacean tissues stranded in Catalonia during the inter-epizootic years would support the hypothesis that CeMV has not circulated in the Mediterranean Sea between the 1990 and the 2007 morbillivirus epizootics.

5. As had occurred after the 1990 epizootic, in the nearby years following the 2007 morbillivirus epizootic the western Mediterranean striped dolphins have suffered a chronic morbillivirus infection, different from the classic systemic form observed during the epizootics, characterized by restriction of lesions and virus to the brain. The mortality associated to this form of the disease was high, showing a decreasing trend.

6. The chronic form of CeMV infection would have similarities with subacute sclerosing panencephalitis (SSPE) and old dog encephalitis (ODE), chronic diseases caused by Measles virus (MV) and Canine distemper virus (CDV) in humans and dogs, respectively. However the dolphin morbillivirus chronic form should be deeply investigated, beyond our first approach, in order to clarify the pathogenesis and the mechanisms of viral persistence in this disease.

APPENDIX

Morbillivirus surveillance work performed with striped dolphin (*Stenella coeruleoalba*) samples received from January 2011 to April 2014 at the Diagnostic Pathologic Service in the Veterinary Faculty of Barcelona

1. Necropsy and sampling

From January 2011 to April 2014 the Diagnostic Pathologic Service at the Veterinary Faculty in Barcelona received samples from 68 striped dolphins found stranded in a state of good preservation in Catalonia, Valencia Region and Andalusia (22, 7 and 39 dolphins, respectively). Necropsy and sampling was done by local organisations (conservation and research groups) working in these regions. The dolphins stranded on the Catalonian coast were necropsied at the Veterinary Faculty of Barcelona and formalin-fixed and frozen tissues were available for study. Formalin-fixed tissues and frozen tissues were received from the dolphins stranded on Valencia Region, with the exception of one case from which only frozen tissues were sent. Formalin-fixed, paraffin-embedded tissue sections in Polysine slides (Thermo Scientific®) were received from the striped dolphins stranded in Andalusia to perform immunohistochemistry for morbillivirus. Brain location sampling was not homogeneous. Formalin-fixed tissues were embedded in paraffin and routinely processed to perform Haematoxylin and Eosin staining.

2. Immunohistochemistry

Immunohistochemical detection of morbillivirus was performed in 53 striped dolphins. The following organs considered target of morbillivirus infection in striped dolphins (Domingo et al. 1992) were studied: lung (50/53), lymphoid tissues (prescapular lymph node, pulmonary lymph node, mesenteric lymph node and/or spleen; 48/53) and brain (52/53). A mouse monoclonal antibody against the canine distemper virus nucleoprotein (Ref. NP 050505 VMRD, Pullman, WA, USA) known to react with CeMV, was used as primary antibody at a dilution of 1:200 in tris-buffered saline (TBS) with 2% bovine albumin. The technique was performed as previously described (Raga et al. 2008). Lung sections from a CeMV infected dolphin were used as positive control in each test. Duplicate tissue sections were also incubated with TBS as the first layer as negative control.

3. Molecular detection of DMV

Molecular detection of DMV was performed by RT-PCR on tissues from 25 striped dolphins. From all these cases, the following organs considered target of CeMV infection (Domingo et al. 1992) were investigated: lung (23/25), lymphoid tissues (prescapular lymph node, pulmonary lymph node, mesenteric lymph node and/or spleen; 23/25) and brain (25/25).

Approximately 1 g of tissue was homogenised in 10 ml of DMEM. The RNA was extracted from tissue homogenates using the RNA viral isolation kit Nucleospin II according to the manufacturer's instructions (Macherey-Nagel Lab.).

DMV molecular detection was performed in all samples by a 1-step RT-PCR technique that amplified a 78 bp fragment (Krafft et al. 1995, Saliki et al. 2002) within the phosphoprotein (P) gene. One negative control and one DMV positive control provided by M. Baron (Institute for Animal Health, Pirbright Laboratory, UK) were included.

4. Epidemiological and pathological results

Monitoring of striped dolphins stranded from January 2011 to April 2014 using histopathology, immunohistochemistry and RT-PCR showed five out of 68 (7.4%) striped dolphins affected by the CeMV chronic infection form, and two out of 68 (2.9%) striped dolphins affected by the CeMV systemic infection form.

The annual incidence of dolphins affected by the chronic form of morbillivirus infection in the three studied regions (Catalonia, Valencia Region and Andalusia) is shown in Table 1. In 2013 zero dolphins out of 18 studied striped dolphins have shown morbillivirus infection. In 2014 we have received only samples from one striped dolphin stranded in Catalonia. It was also negative for morbillivirus.

The five dolphins affected by the chronic form of the morbillivirus infection showed relevant lesions only in the brain. The brain presented non-suppurative meningoencephalitis of variable intensity, with mononuclear perivascular cuffs, gliosis, glial nodules with neuronophagia and spongiosis. Cytoplasmic or nuclear eosinophilic inclusions were occasionally detected. Syncytial cells were not observed. Morbillivirus antigen was found only in brain tissue, mainly in neurons and also in glial cells. Lung and lymphoid tissue

samples were negative. Frozen tissues were available from two dolphins, one from Catalonia and one from Valencia Region. The RT-PCR study in these dolphins showed similar results to the immunohistochemistry, detecting DMV nucleic acid only in brain tissue, with negative results in splenic and lymph node tissues.

The two striped dolphins showing the systemic morbillivirus infection were adult animals, one found in Línea de la Concepción (Cádiz, Andalusia) on the 17th December 2011, and one in Tarifa (Cádiz, Andalusia) on the 22nd August 2012. Both towns are close and are located in the Strait of Gibraltar, where the Mediterranean Sea and the Atlantic Ocean converge. The first dolphin showed intense morbillivirus immunostaining in lung, spleen and brain. Syncytial cells could be also observed in the pulmonary tissue. The second dolphin showed abundant morbillivirus antigen in lung, lymph node and brain, with visible interstitial pneumonia and syncytial cells in the lung and the lymph node samples. Frozen tissue samples from these dolphins were not available for the performance of the RT-PCR study.

One of the striped dolphins studied in this period showed lesions associated to *Toxoplasma gondii* infection, consisting of multifocal granulomatous meningoencephalitis with intralesional parasitic cysts. This dolphin was found on the Cádiz coast (Andalusia) on the 27th October 2011, less than two months before the appearance in the same area of a striped dolphin suffering systemic morbillivirus infection. The dolphin suffering toxoplasmosis was negative for the morbillivirus immunohistochemistry performed in brain, lung and lymph node tissue samples.

Table 1. Summarized data of the striped dolphins (*Stenella coeruleoalba*) stranded on the Spanish Mediterranean coast from January 2011 to April 2014, both by region and year. Each cell represents the number of dolphins showing chronic morbillivirus infection by dolphins tested. The percentage of chronic cases is included in brackets.

LOCATION	2011	2012	2013	2014 (January- April)	TOTAL
Andalusia	0/13 (0)	2/9 (22,2)	0/17 (0)	0	2/39 (5.1)
Valencia Region	1/7 (14.3)	0	0	0	1/7 (14.3)
Catalonia	2/5 (40)	0/15 (0)	0/1 (0)	0/1 (0)	2/22 (9.1)
TOTAL	3/25 (12)	2/24 (8.3)	0/18 (0)	0/1 (0)	5/68 (7.4)

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