TESIS DOCTORAL

STRESS CARDIOMYOPATHY IN CETACEANS

HISTOLOGICAL, HISTOCHEMICAL, IMMUNOHISTOCHEMICAL AND BIOCHEMICAL STUDIES

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La Comisión Académica del Programa de Doctorado, en su sesión de fecha ***/ /*** tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada **"Stress Cardiomyopathy In Cetaceans: Histological, Histochemical, Immunohistochemical and Biochemical Studies"** presentada por la doctoranda **Dª Nakita Câmara** y dirigida por el **Doctor Pedro Herráez Thomas** y por la **Doctora Eva Sierra Pulpillo**.

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

"EL OCÉANO DE LA VIDA"

"Nunca sabes cuándo será la última vez que respires.

Todo puede cambiar en menos de lo que acabas de leer esta página.

Por eso coge aire y sumérgete.

El océano te está esperando y la vida también."

David Sadness

This PhD thesis is dedicated to my lost loved ones, specially my aunt Sandra Miranda, who watches for me in the other side. You will always be loved, never forgotten and forever missed.

I also dedicate this work to my loving parents, Lena and Zé, and courageous sister Angie. As Calum Scott says in one of his song:

“She/He said I love you no matter what

I just want you to be happy and always be who you are

She/He wrapped his arms around me

Said, "Don't try to be what you're not

'Cause I love you no matter what"

She/He loves me no matter what

They love me no matter what”

And finally, to Jesus, the one I love, who showed me that when two people really care about each other, they always find a way to make it work. No matter how hard it is.

The best kind of people are the ones that come into your life and make you see the sun where you once saw clouds. The people that believe in you so much, you start to believe in yourself too. The people that love you, simply for being you. The once in a lifetime kind of people. Therefore, I would like to transmit my gratitude to some extraordinary people, who made this work possible, not only as the result of my own efforts but also of theirs, specially to:

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- The Ministry of Science, Innovation and Universities, subsided by the Government of Gran Canaria, who partially funded this research.

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– Ana, my first mentor: I will never be done thanking you for starting me on the cetacean necropsies and for your constant friendship. I still remember I felt extremely blessed when you accepted to be my master's thesis advisor. I will never forget the words you said to me at that time: "Be careful, I am very demanding!" to which I replied, "That's good, that's what I need!", because as Socrates said: "Under the direction of a strong general, there will never be weak soldiers." I hope I haven't disappointed you so far because the influence of a teacher can never be erased! And as Colleen Wilcox said “it takes a big heart to help shape little minds”, and you definitely helped shaping mine so far.

Furthermore, I would like to recognize the important part of a friend in our lives. A friend is someone we turn to when our spirits need a lift. A friend is someone we treasure, for friendship is a gift. A friend is someone who fills our lives with beauty, joy and grace. A friend turns the world we live in a better and happier place. So, thank you Anna Maria, Cristian, Francesco, José Angel, Maria Carolina, Marina, Marisa, Natalia, Raquel, Tania, Yania, Maria and Fede, Cristina and family, and Maria for being my friends and basically my “Canarian family”, and also to my (actual and future) borrowed nieces (Africa and Carmen) and nephews (Iván, Mateo, Lucas and Thiago), who always puts a smile on my face.

As Thomas Aquinas said, “there is nothing on this earth more to be prized than true friendship” and as Henry David Thoreau “nothing makes the earth seem so spacious as to have friends at a distance; they make the latitudes and longitudes”. Therefore, I would also like to express my gratitude to my friends around the world. To Nicole, from the White Shark Projects, and to Che Rie, from Bayworld in South Africa. To Andrea, Bella, Isabella, Mar (and her little Samuel) Marta, Oihane and Raquel del Solar, or as we call her “Mujer”, friends that I met while working at IUSA. To Gabby, Lili, Luisa, Sofia and João, or as we call him “Rato”, friends from my university in Portugal. And finally, to André (my Valentim) and to my oldest friend Vasco.

And as the last are said to be the first ...

I am eternally grateful to all the members of my family, my constant source of love and inexhaustible understanding, who always encourage me with driving words of confidence, showing me their admiration, emotional support and unwavering enthusiasm. As Brad Henry said: “families are the compass that guide us. They are the inspiration to reach great heights, and our comfort when we occasionally falter”.

First, to Fátima, Marlene and Joana, mother and sisters not by blood but by heart, who have always been present in both my life and my family's life, and for often checking in on mom and dad since me and my sister were not there.

Second, to Micas, my "mini me", for helping me in the lab when you came to visit me and for assisting mommy and daddy with the technological issues for the drawings inside this thesis.

Third, to Tojo (my brother-in-law and "Mr. Engineer" as I call him) for goofing around when we spoke on the telephone and for being patient with my sister when she was helping me.

Unconditionally, to Angie (my sister and my hero), Lena (my mommy) and Zé (my daddy and my "Cuddly cuddle" as I call him), I could never thank you enough for listening to me, for protecting me from the things I shouldn't do, for guiding me in the right direction, for putting up with my mood swings and arrogance, for being there for me without a doubt. I know I never tell you enough how much I love you. No matter what, I always will love you, no matter how much we argue, or how much I upset you, I'll love you till the day I die. I hope that the final result of this work will be worthy of the help (with the English corrections and all the drawings of the cover and each separator sheet), effort and trust you have all placed in me. Without you the realization of it would not have been possible.

And finally, to Jesus, I may not be able to tell you every day but I just want you to know that you mean the world to me. The day you stepped into my life, you changed it into something really beautiful and meaningful. You hear my pain when everyone else ignores it. You always try to make me smile when I think I can't. You listen to my secrets and make them yours. You give me a hug when I can't find my voice. You wipe away the tears that the world makes me weep. You mean more to me than you'll ever know. You're my best friend. You're my safe harbour. I can truly say that without the inspiration, drive, and support that you have given me in these past few years, this work, probably, would not be finished and I would not be the person and fighter I am today.

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ABREVIATURAS
&
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AMI – Infarto Agudo de Miocardio / Acute Myocardial Infarction

BUN – Nitrógeno Ureico en Sangre/ Blood Urea Nitrogen

CK – Creatina Quinasa / Creatine Kinase

CM – Miopatía de Captura / Capture Myopathy

cTnI – Troponina I Cardíaca / Cardiac Troponin I

cTnC – Troponina C Cardíaca / Cardiac Troponin C

ECG – Electrocardiografia / Electrocardiography

HE – Tinción Hematoxilina y Eosina / Haematoxylin & Eosin Stain

MO – Microscópio óptico / Optical Microscope

PAS – Tinción Ácido Periódico de Schiff / Periodic Acid Shiff Stain

PTAH – Tinción Hematoxilina Ácida Fosfotúngstica / Phosphotungstic Acid Haematoxylin Stain

SCMP – Cardiomiopatía por Estrés / Stress Cardiomyopathy

NORMATIVA Y ADECUACIÓN A TESIS DOCTORAL POR COMPENDIO DE PUBLICACIONES

El Reglamento de estudios de doctorado de la Universidad de Las Palmas de Gran Canaria, aprobado en Consejo de Gobierno de 26 de febrero de 2019 (Boletín Oficial de la ULPGC de 04/03/2019), establece en el artículo 12 los requisitos generales que debe cumplir una tesis doctoral por compendio de publicaciones:

1. Para la presentación de tesis por compendio de publicaciones será necesario:
   a) Un mínimo de tres publicaciones, con unidad temática, indexadas en el *Journal Citations Reports*, *Arts* and Humanities Citation Index o equivalentes, de las que el doctorando sea el primer autor o autor principal. Al menos una de ellas deberá haber sido publicada en una revista cuyo índice de impacto la sitúe dentro de la primera mitad en orden decreciente de índice de impacto entre las revistas del área.
   b) Para acreditar la condición de autor principal, esta deberá ser reconocida por el resto de los autores de las publicaciones presentadas como núcleo de la tesis doctoral, al mismo tiempo que estos deberán renunciar a utilizar estas publicaciones como núcleo principal de otras tesis doctorales, sin perjuicio de que dichas publicaciones puedan ser presentadas como méritos complementarios en las tesis doctorales que pudieran presentar los otros autores de dichas publicaciones.
   c) En áreas de especial incidencia tecnológica dos de estas publicaciones podrán ser sustituidas por patentes en explotación o publicaciones en congresos reconocidos por la ANEP en sus baremos para la obtención de sexenios.
   d) Que en las publicaciones o patentes conste la ULPGC a través de la filiación del director o del doctorando.
2. Las tesis doctorales presentadas como compendio de publicaciones deberán ajustarse al formato establecido en los apartados del 1 al 3, del artículo 11 del presente Reglamento y contener los apartados siguientes:
   a) Una introducción en la que se presenten los objetivos de la tesis, los trabajos publicados y la justificación de la unidad temática de la tesis.
   b) Una copia de los trabajos publicados.
   c) Las conclusiones finales.
   d) En el caso de que lo dispuesto en los apartados a y c se haya redactado en una lengua diferente del español, deberá incluirse un resumen en español según el artículo 10 del presente reglamento, de una extensión de entre 5 y 20 páginas, en el que se incluyan los objetivos y las conclusiones.

Esta tesis doctoral cumple con lo establecido en el artículo mencionado anteriormente, ya que se presentan cuatro publicaciones, teniendo todas estas a la doctoranda como primera autora y están publicadas en revistas científicas del ámbito de conocimiento del programa de doctorado. Las cuatro publicaciones cuentan con los indicios de calidad que exige el programa de doctorado, esto es: los cuatro artículos están indexados en *Journal Citations Reports*.

A continuación, se detallan los indicios de calidad de las publicaciones:

1. **Nakita Câmara**, Eva Sierra, Carolina Fernández-Maldonado, Antonio Espinosa de los Monteros, Manuel Arbelo, Antonio Fernández, Pedro Herráez. "Stress cardiomyopathy in stranded cetaceans: a histological, histochemical and immunohistochemical study". doi: 10.1136/vr.105562. Veterinary Record. Impact Factor 2018: 2.101. Journal Rank in Veterinary Sciences category: 16/141 (Q1).

2. **Nakita Câmara**, Eva Sierra, Antonio Fernández, Manuel Arbelo, Marisa Andrada, Antonio Espinosa de los Monteros, Pedro Herráez. "Increased plasma cardiac troponin I in live-stranded cetaceans: correlation with pathological findings of acute cardiac injury". doi: 10.1038/s41598-020-58497-3. Scientific Reports. Impact Factor: 4.011. Journal Rank in Multidisciplinary Sciences category: 15/69 (Q1).

3. **Nakita Câmara**, Eva Sierra, Antonio Fernández, Cristian Manuel Suárez-Santana, Raquel Puig-Lozano, Manuel Arbelo, Pedro Herráez. "Skeletal and cardiac rhabdomyolysis in a live-stranded neonatal Bryde's whale with fetal distress". doi: 10.3389/fvets.2019.00476. Frontiers in Veterinary Science. Impact Factor: 2.029. Journal Rank in Veterinary Sciences category: 18/141 (Q1).

4. **Nakita Câmara**, Eva Sierra, Antonio Fernández, Manuel Arbelo, Yara Bernaldo de Quirós, Marina Arregui, Francesco Consoli, Pedro Herráez. "Capture Myopathy and Stress Cardiomyopathy in a Live-Stranded Risso's Dolphin (Grampus griseus) in Rehabilitation". doi: 10.3390/ani10020220. Animals. Impact Factor: 1.832. Journal Rank in Veterinary Sciences category: 29/141 (Q1).

En conformidad con lo establecido en el artículo 12 del Reglamento de estudios de doctorado para la presentación de tesis por compendio de publicaciones, se muestra la unidad temática de las publicaciones aportadas. La unidad temática de las publicaciones que integran

esta tesis por compendio es manifiesta, pues todas versan sobre la Cardiomiopatía por Estrés en cetáceos, habiendo abordado esta patología a través del estudio bioquímico, histológico, histoquímico e inmunohistoquímico.

Finalmente, la estructura de esta tesis doctoral cumple lo establecido en el artículo 12.2 del Reglamento de estudios de doctorado, como puede comprobarse en las páginas siguientes, pues ofrece:

a) una introducción en la que se presenta los objetivos de la tesis, la presentación de los trabajos publicados y la justificación de la unidad temática de la tesis (revisión bibliográfica);
b) una copia de los trabajos publicados (publicaciones);
c) las conclusiones finales.

RESUMEN

1.1 ANTECEDENTES & OBJETIVOS DEL ESTUDIO

Estudios previos han puesto de manifesto la susceptibilidad de los cetáceos al estrés, a través de análisis de sangre, que demuestran la activación del eje Hipotalámico-Hipofisario-adrenal con la consiguiente producción y liberación de glucocorticoides, mineralocorticoides y catecolaminas (adrenalina y noradrenalina) (Prosser 1986; Spraker 1993).

Aunque los efectos beneficiosos de la activación del sistema de estrés son bien conocidos, los animales pueden verse afectados negativamente por los efectos del estrés físico y psicogénico en situaciones extremas o prolongadas (Cowan and Curry 2002).

Por lo tanto, los mamíferos marinos pueden desarrollar respuestas extremas frente al estrés que causan un deterioro notable en la salud e incluso la muerte. Las lesiones cardíacas parecen jugar un papel central en estas respuestas adversas al estrés en los cetáceos, y diferentes autores sugieren que estos animales podrían estar particularmente predispuestos a desarrollar cardiomiopatía por estrés (SCMP) probablemente debido a sus adaptaciones cardiovasculares al metabolismo del buceo (Cowan and Curry 2002; Cowan, Harter, and Kandel 2000; Cozzi, Huggenberger, and Oelschläger 2017; Herráez et al. 2007, 2013).

En las últimas décadas, se han realizado esfuerzos para reducir el impacto que algunas actividades humanas tienen en los cetáceos de vida libre, pero desafortunadamente las ballenas y los delfines continúan amenazados, entre otros, por el varamiento activo (vivo) y posterior interacción con los humanos, las colisiones con embarcaciones y las capturas accidentales (bycatch). Estas entidades patológicas tienen el estrés agudo como eje común de su etiopatogenia, pudiendo causar la muerte del animal o agravar una situación de enfermedad previa, al tiempo que influye en su rehabilitación posterior, haciendo que la terapia y la recuperación de los animales involucrados no tengan éxito en la mayoría de las ocasiones (Arbelo et al. 2013; Bonsembiante et al. 2017; Cowan and Curry 2008; Díaz-Delgado et al. 2018; Duignan and Jones 2005; Gulland, Dierauf, and Whitman 2018; Herráez et al. 2007, 2013; Sierra et al. 2014, 2017; Soulsbury, Iossa, and Harris 2008).

El presente trabajo ha sido diseñado con el objetivo de continuar la investigación en cetáceos con una doble intención: por un lado, caracterizar la cardiomiopatía por estrés, tratando de proporcionar un mayor conocimiento sobre las entidades patológicas causantes y, por otro lado, aplicar este conocimiento a soluciones concretas, utilizando la salud como un instrumento necesario para la conservación de estos animales en nuestros mares.

Estudios anteriores muestran que, en las Islas Canarias desde el año 1999 hasta el año 2005, el 27% (37 animales de un total de 138) de los animales vararon activamente y, en el período comprendido entre el año 2006 al 2012, el 13% (30 animales de un total de 236). Además, en Andalucía entre el año 2011 y el 2014, el 27% (28 animales de un total de 104) de los animales estaban vivos cuando vararon (Arbelo et al. 2013; Díaz-Delgado et al. 2018; Maldonado 2015). Por lo tanto, es extremadamente importante estudiar los parámetros bioquímicos relacionados con la SCMP en los cetáceos, ya que es de nuestro interés poder reintroducir al medio marino estos animales con el mínimo daño posible. Del mismo modo, la medición de estos parámetros en el momento del varamiento y el uso de medicamentos que controlan los efectos negativos de la liberación de altas concentraciones de catecolaminas al torrente sanguíneo, responsables entre otros del daño cardíaco.

Así, y con estos antecedentes, el objetivo general de este estudio ha sido caracterizar la SCMP bioquímica y morfológicamente, como lesiones resultantes de respuestas extremas al estrés en cetáceos varados activamente y sometidos a manipulación e interacción con humanos, cetáceos muertos debido a colisiones con barcos e interacción con actividades pesqueras (bycatch).

Esto se logrará a través de los siguientes objetivos específicos:

1. Caracterizar la cardiomiopatía por estrés (SCMP), como lesión resultante de las respuestas al estrés extremo en los cetáceos que han varados activamente y han sido sometidos a manipulación e interacción con humanos, que han muerto tras la colisión con una embarcación o como consecuencia de interacciones pesqueras (captura accidental o bycatch).
2. Correlacionar los valores sanguíneos de los marcadores bioquímicos relacionados con el daño muscular cardíaco agudo con los hallazgos patológicos identificados histológicamente, en cetáceos varados activamente y sometidos a manipulación e interacción con humanos.
3. Aplicar los conocimientos obtenidos en el diagnóstico de casos clínicos específicos.

1.2 MATERIALES & MÉTODOS

a) Animales incluidos en el estudio

Para realizar este estudio, utilizamos muestras de corazón de cetáceos (ballenas y delfines) varados en diferentes localizaciones geográficas del archipiélago canario y Andalucía. Estos tejidos se almacenan en el banco de tejidos del Centro Atlántico de Cetáceos, División de Histología y Patología Animal, del Instituto Universitario de Sanidad Animal y Seguridad Alimentaria, perteneciente a la Universidad de Las Palmas de Gran Canaria (ULPGC).

Para cada objetivo específico de este trabajo se realizó una selección de muestras concretas, la cual se explica a continuación:

i. Caracterización de la cardiomiopatía por estrés en los cetáceos

La primera fase de este objetivo específico consistió en la selección de los animales del estudio (n=148), incluyendo los que vararon activamente y posteriormente murieron antes o durante el rescate y la rehabilitación (n=83), los que murieron debido a la colisión con una embarcación (n= 32), y los que murieron atrapados en redes de pesca (n=33). Todos estos animales aparecieron varados en las costas de las Islas Canarias entre los años 2000 y 2016 y en las costas de Andalucía entre 2011 y 2014.

Posteriormente, del total de animales se seleccionaron 67 (48 varados activamente, 7 muertos por colisión con embarcaciones y 12 por captura accidental) en función de su estado de conservación, descartándose aquellos que se encontraban en un estado de autolisis avanzada (grado 4) o muy avanzada (grado 5). Igualmente se descartaron aquellos animales que habían sido congelados previamente a la realización de la necropsia ya que generan falsos negativos al usar técnicas histoquímicas e inmunohistoquímicas (ASCOBANS/ACCOBAMS 2019; Godinho 2010).

ii. Correlación de los valores sanguíneos de los marcadores bioquímicos relacionados con el daño muscular cardíaco agudo con los hallazgos patológicos identificados histológicamente

La primera fase de este objetivo específico consistió en la determinación de valores normales de troponina I cardíaca (cTnI) en cetáceos, específicamente en delfines mulares (n=5) pertenecientes a un zoológico local (Palmitos Park). Para lograr esto, se recolectaron un total de

20 muestras de sangre en los meses de junio, septiembre y diciembre de 2018; así como en marzo de 2019 (4 muestras de cada individuo, 1 muestra por mes). Para calcular el intervalo de referencia, que permite establecer un rango de valores mínimo y máximo dentro de los cuales se encuentra el 95% de los valores observables de la variable en una población sana de referencia, se realizó una estimación de la variabilidad intersujetos mediante el método de estimación de componentes de la varianza descrito en Rasch and Mašata (2006), y se asumió distribución normal en las variables para construir los intervalos de referencia tal como se describe en Friedrichs et al. 2012.

En la segunda fase, se incluyeron en este estudio un total de 9 animales que vararon activamente. Se recogieron muestras de sangre de estos animales, siendo una de las muestras tomadas ante-mortem y el resto post-mortem debido a la muerte de los animales antes o durante la manipulación, transporte y/o rescate/rehabilitación (n=8). Todos estos animales vararon en las costas de las Islas Canarias desde principios de 2016 hasta junio de 2019.

Durante la tercera fase, se seleccionaron un total de 7 animales, de los animales incluidos (n=9) en la fase anterior, para el estudio histológico e histoquímico, ya que no pudimos realizar la necropsia en 2 animales, porque uno fue liberado de vuelta al mar y otro fue utilizado con fines de investigación anatómica.

En la cuarta fase, 2 animales (de la fase anterior) fueron eliminados del estudio debido a su congelación previa. Por lo tanto, al final de esta fase, se estudiaron un total de 5 individuos con el uso de la técnica inmunohistoquímica para la detección de diferentes marcadores.

iii. Aplicación de los conocimientos obtenidos en el diagnóstico de casos clínicos específicos

Para lograr este objetivo específico, se seleccionaron cuidadosamente 2 animales en función de características específicas.

El primer animal fue seleccionado en base al hecho de que era un ejemplar, que varó activamente, perteneciente al infraorden *Mysticeti*, ya que la descripción de la SCMP en estas especies es aún escasa.

El segundo caso de estudio se centra en un animal varado activamente que estuvo en rehabilitación durante 6 días, lo que nos permitió obtener muestras de sangre consecutivas *in vivo*.

b) Técnicas utilizadas en el estudio

i. Recogida de las muestras de sangre

Se obtuvieron muestras de sangre de la aleta caudal de cada animal que fueron depositadas en tubos sin anticoagulante. Posteriormente, las muestras fueron centrifugadas a 3500 rpm durante 5 minutos, dos veces para obtener el suero (aproximadamente 1 ml).

ii. Necropsia

Se realizó el examen postmortem completo siguiendo el protocolo estándar publicado por la European Cetacean Society, con algunos procedimientos adicionales detallados en el manual *Marine Mammals Ashore: A Field Guide for Strandings* (Geraci and Lounsbury 2005; Kuiken and Hartmann 1991). El estado de conservación de los animales también se determinó siguiendo los parámetros y clasificaciones establecidas por estos dos protocolos.

Muestras de tejido representativas de cada órgano se fijaron en formalina al 10% durante aproximadamente 48h y se procesaron utilizando el protocolo estándar. Fueron colectadas y posteriormente analizadas muestras de músculo cardíaco (tanto aurículas como ventrículos), válvulas auriculoventriculares (bicúspides o mitrales y tricúspides), válvulas semilunares (sigmoide aórtico y sigmoide pulmonar con las correspondientes arterias). En los casos clínicos específicos, debido a la posible presencia de CM, los músculos esqueléticos (*longissimus dorsi* y *rectus abdominis*) y los riñones fueron también analizados.

iii. Técnicas Histológicas & Histoquímicas

Se realizaron secciones de tejido (4 µm de grosor) para la tinción de hematoxilina y eosina (HE) y tinción de ácido periódico de Schiff (PAS), mientras que se utilizaron cortes de 5 µm de grosor para las técnicas de hematoxilina ácida fosfotúngstica (PTAH) y tricrómico de Masson.

iv. Técnica Inmunohistoquímica

Las secciones de tejido (3 µm de grosor) se inmunomarcaron con los anticuerpos anti-mioglobina (1:200), anti-fibrinógeno (1:50), anti-troponina I cardíaca (1:25) y anti-troponina C cardíaca (1:250). Los anticuerpos primarios fueron visualizados usando el VECTASTAIN Kit Elite ABC-Peroxidase (PK-6100) de Vector Laboratories (Peterborough, Reino Unido).

El control negativo consistió en secciones en serie de corazón sin el anticuerpo primario. Por el contrario, el control positivo de mioglobina y fibrinógeno provenía de una muestra de corazón de delfín listado (*Stenella coeruleoalba*). El delfín había varado activamente y desarrolló miopatía de captura (CM) debido a la manipulación en sí y a la interacción humana durante el proceso de rehabilitación (Herráez et al. 2007, 2013). Finalmente, se usaron muestras de corazón de un cerdo y un cetáceo, sin lesiones macroscópicas y/o histológicas, como controles positivos para la troponina I y troponina C (cTnC).

1.3 RESULTADOS Y DISCUSIÓN: PUBLICACIONES

a) Caracterización de la cardiomiopatía por estrés en los cetáceos

El primer objetivo específico de este trabajo, que consistió en caracterizar la SCMP en los cetáceos como lesiones resultantes de respuestas de estrés extremo, se logró mediante análisis histológicos, histoquímicos e inmunohistoquímicos en un total de 67 animales que vararon activamente (n=48) o murieron debido a colisiones con embarcaciones (n=7) e interacciones con pesca (bycatch) (n=12).

Histológicamente, las principales lesiones cardíacas asociadas con el estrés fueron cambios vasculares, que se presentaron como congestión, hemorragias y edema intersticial; lesiones degenerativo-necróticas cardíacas agudas o subagudas, que se atribuyeron a la cardiotoxicidad por las catecolaminas y consistieron en necrosis en banda de contracción, fibras en acordeón, hipereosinofilia citoplasmática y vacuolización perinuclear; la presencia de glóbulos de mioglobina intersticiales y la infiltración de células inflamatorias. Estos hallazgos coinciden con los descritos en análisis histológicos de biopsias y/o de tejido miocárdico post-mortem de humanos con SCMP (Akashi et al. 2010; Fineschi et al. 2010; Jaspreet, Wangde, and A. 2019; Jiang and Downing 1990; Kawai 2012; Maréchaux et al. 2008; Mitchell and Marquis 2017; Miura et al. 2017; Pascual, Abó, and Piqué 2015; Prasad, Lerman, and Rihal 2008).

La congestión subendocárdica, el edema intersticial y las hemorragias se detectan mediante abordajes histológicos y forman parte de la SCMP (Jiang and Downing 1990).

Se observó congestión vascular en 45 de los 67 animales (67,2%); hallazgo vascular que está frecuentemente presente en situaciones estresantes (Cowan and Curry 2008; Díaz-Delgado et al. 2018; Fishbein 2001; Herráez et al. 2007, 2013).

Las muestras de tejido de 26 animales (38.8%) contenían fibras separadas por espacios claros, que representan edema intersticial (Jaspreet et al. 2019). Después de la administración de catecolaminas, el edema intersticial generalmente se asocia con hemorragias subendocárdicas y subepicárdicas. El edema intersticial está característicamente presente en las áreas dañadas del miocardio, incluso después de 72 horas de actuar la causa desencadenante (Fishbein 2001). En nuestro estudio, detectamos edema y hemorragias en 8 de los 67 animales analizados (11,9%).

Las hemorragias subendocárdicas focales y difusas son visibles en el examen macroscópico. Estas lesiones también se detectan en ocasiones en el miocardio y el epicardio, poco después de la administración y/o liberación de grandes dosis de catecolaminas en humanos con SCMP (Jiang and Downing 1990; Mitchell and Marquis 2017). Quince animales (22,4%) presentaron hemorragias de localización epicárdica y/o subendocárdica detectables tanto macro como microscópicamente. Estos hallazgos coinciden con estudios previos centrados en muertes agudas asociadas con el estrés, particularmente en animales que murieron después de un varamiento activo y subsecuente manipulación/rehabilitación/recuperación (Díaz-Delgado et al. 2018; Herráez et al. 2007, 2013).

La necrosis en banda de contracción se desarrolla entre los 5 a 10 minutos después de un episodio de isquemia transitoria y reperfusión. Es una lesión músculo-esquelética y miocárdica característica asociada con la administración de catecolaminas, o con altas concentraciones de catecolaminas endógenas (Fineschi et al. 2010; Reichenbach and Benditt 1970; Turnbull and Cowan 1998). Es, por tanto, una lesión que se describe ampliamente como un indicador de SCMP. La necrosis en banda de contracción ha sido descrita en humanos como consecuencia de eventos estresantes (Akashi et al. 2010; Fineschi et al. 2010; J. et al. 2008; Jiang and Downing 1990; Kawai 2012; Maréchaux et al. 2008; Mitchell and Marquis 2017; Miura et al. 2017; Pascual et al. 2015; Prasad et al. 2008), así como en otros animales, como focas (Seguel et al. 2014) y cetáceos, debido a muertes agudas como las producidas por colisiones con barcos, captura accidental, y/o varamientos activos con subsecuente estrés por el manejo (Arbelo et al. 2013; Cowan and Curry 2002, 2008; Díaz-Delgado et al. 2018; Herráez et al. 2007, 2013; Sierra et al. 2014). En nuestro estudio, 33 animales presentaron necrosis en banda de contracción (49.3% de todos los animales y 100% de los animales que murieron por colisión con embarcación, 52.1% de los animales varados activamente y 8.3% de los animales que murieron por la captura accidental). Esta condición fue más marcada a nivel subendocárdico y

subepicárdico. El daño fue más severo en los animales que murieron por colisiones con barcos, lo que coincide con estudios previos (Adegboyega, Haque, and Boor 1996; Sierra et al. 2014).

El primer hallazgo histológico asociado a la isquemia es la presencia de fibras onduladas largas y adelgazadas, denominadas fibras en acordeón (Fineschi et al. 2010; Fishbein 2001). Este hallazgo se detectó en 29 animales presentes en nuestro estudio (43.3%). Esta lesión ha sido previamente descrita en animales que murieron súbitamente tras un episódio estresante (Cowan and Curry 2002, 2008; Herráez et al. 2007, 2013). Es probable que las fibras onduladas resulten de las fuerzas sistólicas de las fibras viables inmediatamente adyacentes a las fibras muertas no contráctiles (Fishbein 2001; Kumar et al. 2015). Aunque las fibras en acordeón no se consideren fibras en degeneración y/o necrosis si pueden ser usadas como un indicador temprano de lesión miocárdica isquémica. Este hecho ha sido reproducido experimentalmente tras la oclusión de las arterias coronarias (Fishbein 2001).

La hipereosinofilia es el primer cambio confirmado específico de la necrosis miocárdica utilizando técnicas histológicas (HE) e histoquímicas (PTAH y el tricrómico de Masson). Consiste en un aumento de la tinción eosinofílica (HE), azul (PTAH) o roja (tricrómico), respectivamente, de los cardiomiocitos necróticos (Fineschi et al. 2010; Fishbein 2001). En nuestro estudio, todos los animales (100%) exhibieron hipereosinofilia citoplasmática. En estudios experimentales, este cambio de color y edema intersticial sutil es evidente 2 a 3 horas después de la oclusión coronaria, y es más pronunciado y detectable a las 3-6 horas (Fishbein 2001). Los animales que mueren debido a una situación estresante presentan este cambio citoplasmático (Cowan and Curry 2002, 2008; Herráez et al. 2007, 2013; Sierra et al. 2014). La hipereosinofilia fue más pronunciada en la zona subendocárdica y subepicárdica, lo que respalda estudios anteriores que mostraron cómo progresa la lesión irreversible en un movimiento de frente de onda desde el subendocardio isquémico. El déficit de perfusión es más grave en el subendocardio ya que el subepicardio recibe un flujo sanguíneo colateral (Buja 2005; Cowan and Curry 2002, 2008).

La degeneración vacuolar se caracteriza, morfológicamente, por la acumulación intracelular de líquido y la lisis de miofibrillas. Este hallazgo se detectó en 65 animales (96,9%) siendo más notable a nivel subendocárdico y subepicárdico. Estudios previos lo detectaron comúnmente en la periferia de los infartos de miocardio y en las regiones subepicárdicas, subendocárdicas y perivasculares que sufren de isquemia grave, mantenida y letal (Adegboyega et al. 1996; Cowan and Curry 2002, 2008). Si bien esta condición es bastante común, las características morfológicas y la importancia funcional de la degeneración vacuolar son poco conocidas. Aunque no se conoce la patogénesis, se hipotetiza que es el resultado del aumento

de la permeabilidad de la membrana celular del miocardio, con el consiguiente flujo de fluidos, inducida por estados hipóxicos. Este fenómeno se observa normalmente en muertes agudas asociadas con situaciones estresantes (Adegboyega et al. 1996; Cowan and Curry 2002, 2008).

Estudios anteriores han demostrado la necesidad de corroborar los hallazgos histológicos con marcadores específicos que podrían exponer mejor el daño al miocardio (Bonsembiante et al. 2017; Herráez et al. 2007, 2013; Seguel et al. 2014). Por lo tanto, en este estudio, comparamos los marcadores que se han utilizado previamente en estudios similares, como la mioglobina y el fibrinógeno (Herráez et al. 2007, 2013; Ortmann, Pfeiffer, and Brinkmann 2000; Sierra et al. 2014; Xiaohong et al. 2002), con los marcadores utilizados en estudios de muestras de corazón humano con SCMP, como la cTnI y la cTnC (Fishbein et al. 2003; Hansen and Rossen 1999; Martínez-Díaz et al. 2005; Ortmann et al. 2000). La depleción de proteínas musculares (esqueléticas y cardíacas) comienza inmediatamente después de un daño celular. Como resultado, se produce una ruptura temprana de la membrana celular del miocardio, lo que provoca una disminución rápida del contenido de mioglobina, cTnI y cTnC, junto con el depósito de proteínas plasmáticas, como el fibrinógeno, en los cardiomiocitos. La tinción homogénea de la mioglobina se observa en el músculo cardíaco normal, mientras que los cardiomiocitos lesionados muestran tanto la depleción de la mioglobina dentro de las fibras musculares como la acumulación intercelular e intersticial de mioglobina (Herráez et al. 2007, 2013; Ortmann et al. 2000; Sierra et al. 2014; Xiaohong et al. 2002). El estudio actual confirmó la pérdida de mioglobina y la acumulación de fibrinógeno, de los cardiomiocitos degenerados en la zona adyacente, así como dentro de la necrosis en banda de contracción.

Este estudio es el primero en utilizar marcadores específicos, como cTnI y cTnC, para detectar daños en el corazón de los cetáceos.

La troponina es un complejo regulador de tres subunidades de proteínas liberadas de los cardiomiocitos, cuando se produce un daño irreversible del miocardio. Las tres subunidades son la Troponina C (componente de unión a calcio), la Troponina T (componente de unión a tropomiosina) y la Troponina I (componente inhibidor). La subunidad cTnI es, en la actualidad, el biomarcador más aceptado, ya que es altamente específico para el tejido cardíaco y proporciona una alta precisión diagnóstica del infarto de miocardio (Lewandrowski, Chen, and Januzzi 2002). Aunque cTnI y cTnC se deplecionan en los cardiomiocitos dañados, como se demostró en nuestro estudio, estos dos marcadores se expresaron intensamente en la necrosis en banda de contracción y en algunas células aisladas que mostraban degeneración. Estudios previos indican que las células que sufren apoptosis tienen una mayor concentración de

troponina debido a la condensación, mientras que los antígenos tisulares se agotan severamente en zonas con necrosis isquémica evidente (zonas de infarto) (Fishbein et al. 2003; Hansen and Rossen 1999; Martínez-Díaz et al. 2005; Ortmann et al. 2000).

Estudios recientes apoyan el concepto de que la patogénesis de la SCMP es causada por isquemia miocárdica aguda. En nuestro estudio, identificamos y describimos los cardiomiocitos dañados (es decir, células individuales o grupos de células dañadas). Estas células comúnmente presentan hipereosinofilia y vacuolización citoplasmática en una distribución multifocal, particularmente en las filas de cardiomiocitos localizados en la periferia de los vasos sanguíneos. Este patrón lesional perivascular, principalmente observado en animales varados activamente, sugiere que la patogénesis de estas lesiones se asocia con fenómenos de isquemia y subsecuente reperfusión. La lesión por isquemia-reperfusión es un fenómeno que se describe como una lesión acelerada en el corazón, debido a que el aporte sanguíneo se reabastece en el área isquémica del corazón que anteriormente estaba privada de su suministro de sangre (isquemia). Como resultado, estas áreas son objeto de hipoxia, pérdida sustancial de la regulación del volumen celular y posterior reentrada de calcio debido al funcionamiento inadecuado de las bombas de iones. El daño a la membrana también ocurre cuando se restablece el suministro de sangre en áreas con células potencialmente viables (Buja 2005; Buja and Butany 2015).

Otro hallazgo anatomopatológico descrito en el examen post mortem y las biopsias endomiocárdicas de pacientes humanos y animales experimentales con SCMP es la infiltración intersticial de linfocitos, neutrofilos y macrófagos (Jaspreet et al. 2019; Mitchell and Marquis 2017; Pascual et al. 2015). En este estudio, se identificaron diferentes tipos de células inflamatorias en 17 animales (25,4%). Estas células se infiltraron en zonas con hemorragias, ruptura fibrilar de fibras, degeneración y necrosis de células cardíacas.

Estos resultados dieron lugar a la publicación de un artículo titulado ***Stress cardiomyopathy in stranded cetaceans: a histological, histochemical and immunohistochemical study***, en *Veterinary Record,* Volume 185, Issue 22 (doi: 10.1136/vr.105562), teniendo esta revista 2.101 de índice de impacto en 2018 y siendo, por eso, una revista Q1 en la categoría de ciencias veterinarias.

b) *Correlación de los valores sanguíneos de los marcadores bioquímicos relacionados con el daño muscular cardíaco agudo con los hallazgos patológicos identificados histológicamente*

El segundo objetivo específico de este trabajo consistió en correlacionar los valores sanguíneos detectados para los marcadores bioquímicos de daño muscular cardíaco agudo [creatina quinasa (CK) y troponina I cardíaca (cTnI)] con los hallazgos patológicos identificados a nivel microscópico.

En humanos, los criterios de diagnóstico de la SCMP comprenden alteraciones en los exámenes médicos (como electrocardiografía, ecocardiografía, cateterismos cardíacos) y análisis bioquímicos. Las anormalidades más comunes a nivel laboratorial consisten en el aumento discreto y temprano de cTnI y/o CK (Fineschi et al. 2010; J. et al. 2008; Lyon et al. 2016).

La enzima sérica más utilizada en la determinación del daño neuromuscular en animales es la creatina quinasa (CK); También se utiliza en la detección de lesiones miocárdicas en humanos (Lewandrowski et al. 2002; Valberg 2008). La CK aumenta a las 3–12 horas después de la lesión muscular, alcanzan sus máximos valores 12–24 horas y vuelve a la línea basal después de 48–72 horas, a menos que se haya producido una nueva lesión o daño permanente (Lewandrowski et al. 2002). Todos los animales analizados en nuestro estudio, y en comparación con la literatura publicada, presentaron valores de CK elevados (animales 1, 3, 5, 6, 7 y 9).

Sin embargo, aunque la CK se considera un marcador sensible de daño miocárdico, también está presente en los músculos esqueléticos en altas concentraciones, así como en el intestino, el diafragma, el útero y la próstata en cantidades menores; por lo tanto, tiene poca especificidad cuando se usa para detectar daño cardíaco agudo (Khan 2015). En consecuencia, las troponinas se han aceptado como el principal marcador en patología humana, ya que cTnI es detectable en cantidades muy bajas (por ejemplo, 0,01 µg/L) en la sangre de individuos sanos sin evidencia de enfermedad cardíaca (Hassan et al. 2009; Lewandrowski et al. 2002; Morrow et al. 2007; Venge et al. 2009). Por lo tanto, se cree que las elevaciones significativas (≥0.1 µg/L) de este marcador probablemente reflejan necrosis miocárdica, y algunos autores lo han descrito como cardioespecífico debido no sólo a su especificidad para el tejido miocárdico, sino también por su alta sensibilidad (Hassan et al. 2009; Lewandrowski et al. 2002; Morrow et al. 2007). Por esta razón, la cTnI se utiliza para detectar varias patologías cardíacas, como el infarto agudo de miocardio (AMI) y la SCMP (Khan 2015; Lewandrowski et al. 2002; Lyon et al. 2016).

En la primera fase de este trabajo, estimamos un rango de referencia para cTnI en cetáceos, ya que no existen datos en la literatura actual. Para lograr esto, se recolectaron un total de 20 muestras de sangre, en junio, septiembre y diciembre de 2018 y marzo de 2019 (4 muestras de cada individuo, 1 muestra por mes) de 5 delfines mulares mantenidos bajo el cuidado humano.

Así, el rango basal obtenido en este estudio y aplicable para cetáceos va desde 0 µg/L (como valor mínimo) hasta 0.025 µg/L (como valor máximo).

Al comparar este rango en particular (0–0.0256 µg/L) con los valores normales para humanos (≤0.1 µg/L) y perros (≤0.03–0.07 µg/L), podemos concluir que el intervalo de referencia para cTnI en delfines mulares es más bajo que en las otras especies (Padilla n.d.; Sleeper, Clifford, and Laster 2001; Wray 2017).

La segunda fase consistió en comparar los valores de 9 cetáceos varados activamente con el rango normal de cetáceos (determinado en la fase previa).

Nuestros resultados, de los cetáceos varados activamente, mostraron que 8 animales [animal 1 (0.06 µg/L), animal 2 (40.00 µg/L), animal 4 (0.235 µg/L), animal 5 (0.249 µg/L), animal 6 (0,748 µg/L), animal 7 (0.033 µg/L), animal 8 (0.06 µg/L) y animal 9 (0.049 µg/L)] presentaron un aumento en comparación con el valor normal/basal para cTnI (0–0.0256 µg/L) que obtuvimos de delfines mulares bajo cuidado humano y que uno de los animales [animal 3 (0.025 µg/L)] presentó un valor de cTnI dentro del rango normal.

En general, los cardiomiocitos lesionados liberan troponina 3–9 horas después del daño isquémico, alcanzan su nivel sérico máximo después de 12–48 horas y permaneciendo elevados durante 4–7 días (Hansen and Rossen 1999; Khan 2015; Lewandrowski et al. 2002). El reconocimiento temprano de la necrosis miocárdica (1–3 horas) no es posible mediante el monitoreo de la troponina sérica, y estos marcadores son ineficaces hasta 6 o más horas después del inicio del AMI y/o SCMP (Hansen and Rossen 1999; Khan 2015; Lewandrowski et al. 2002). La determinación precisa del momento del evento de estrés y/o aparición de síntomas a menudo es excepcionalmente difícil porque se centra en el informe clínico del paciente. Por lo tanto, en humanos, una condición previa para obtener una capacidad satisfactoria para distinguir estas patologías es que la sangre se debe recolectar entre 6 y 9 horas después del inicio de la causa desencadenante (Khan 2015). En el caso de un evento de varamiento activo, a menudo también es clínicamente y patológicamente desafiante conocer el momento exacto del episodio estresante, ya que estamos trabajando con animales salvajes, redes de varamientos y

el público en general. Cuando se notifica el varamiento, es importante reconocer que el animal podría haber varado recientemente o ser detectado mucho tiempo después de haber varado.

Además, estudios previos han propuesto que medir el nivel de troponina en el suero puede ser un método auxiliar importante para examinar la muerte súbita, ya que su concentración máxima puede estar relacionada con el grado de lesión tisular (Khan 2015). Por esta razón, este hallazgo se confirmó con los análisis histológicos, histoquímicos e inmunohistoquímicos (tercera y cuarta fase del estudio).

Una lesión isquémica aguda, como la que ocurre en el AMI o SCMP, se determina por alteraciones morfológicas, que consisten en cambios vasculares y lesiones degenerativas agudas basadas en el análisis histológico del tejido miocárdico (Akashi et al. 2010; Buja 2005; Buja and Butany 2015; Câmara et al. 2019; Cebelin and Hirsch 1980; Fineschi et al. 2010; Jaspreet et al. 2019; Jiang and Downing 1990; Kawai 2012; Lyon et al. 2008; Maréchaux et al. 2008; Mitchell and Marquis 2017; Miura et al. 2017; Pascual et al. 2015; Prasad et al. 2008).

En toda la lesión miocárdica isquémica aguda hay una secuencia cronológica de cambios. Desde los primeros 5 minutos se observa la presencia de fibras adelgazadas y onduladas separadas por espacios que representan edema intersticial y congestión microvascular, localizadas en los bordes del miocardio isquémico. En los siguientes 15 minutos, la muerte celular puede comenzar a ocurrir. Los cambios tempranos de la degeneración y necrosis de coagulación de cardiomiocitos caracterizada por picnosis nuclear, cambio de color, más específicamente "cambio de color rojo ladrillo" o hipereosinofilia citoplasmática, necrosis en banda de contracción focal y edema intersticial sutil, son evidentes dentro de 2-3 horas. La hipereosinofilia y el edema se vuelven más pronunciados y más fácilmente reconocibles después de un período de 3-6 horas. Posteriormente, 6-12 horas más tarde, se observa un mayor número de neutrófilos alineados en los capilares, así como cambios acelerados y una necrosis en banda de contracción más extensa. En el próximo período de 12 horas, ocurre la extravasación en el espacio intersticial de los neutrófilos. También se reconocen congestión vascular, edema intersticial y áreas focales de hemorragia. A partir de entonces, comienza el período subagudo (Buja and Butany 2015).

Teniendo en cuenta todo lo anterior, concluimos que todos los resultados obtenidos de los animales de este segundo estudio estaban de acuerdo tanto con la cinética bioquímica como con la secuencia cronológica de los cambios histopatológicos en una lesión miocárdica isquémica aguda.

Los cardiomiocitos dañados, durante la lesión cardíaca, liberan cTnI y cTnC, lo que resulta en un aumento de los niveles séricos y una disminución de la inmunoreacción de las troponinas en los cardiomiocitos (Khan 2015; Mikaelian et al. 2008). En el presente estudio, la severidad del daño presente en las células se determinó mediante el inmunomarcaje. Todos los animales analizados presentaron depleción tisular de las troponinas I y C cardíacas, así como de mioglobina, junto con la deposición intrafibrilar de fibrinógeno. En consecuencia, con estos cambios inmunohistoquímicos, confirmamos que las lesiones presentes en estos animales eran ante mortem.

Estos resultados fueron publicados en el artículo titulado ***Increased plasma cardiac troponin I in live-stranded cetaceans: correlation with pathological findings of acute cardiac injury***, en la revista *Scientific Reports,* Volume 10, Issue 1 (doi: 10.1038/s41598-020-58497-3), teniendo esta revista 4.011 de índice de impacto en 2018 y siendo, por eso, una revista Q1 en la categoría de ciencias multidisciplinares.

c) *Aplicación de los conocimientos obtenidos en el diagnóstico de casos clínicos específicos*

El tercer objetivo específico de este estudio consistió en la aplicación de los conocimientos obtenidos en el diagnóstico de dos casos clínicos concretos.

i. Caso clínico específico 1

El objetivo principal del análisis forense de los animales de vida silvestre es reconocer los cambios patológicos y la causa de la muerte. Aunque no siempre es posible determinar la enfermedad específica y/o la etiología, la descripción y la posterior interpretación de las lesiones proporcionan unas contribuciones estimables al conocimiento de la patología de los cetáceos. Si bien, los estudios patológicos han sido reportados previamente en varias especies de cetáceos, tales descripciones en el infraorden *Mysticeti* siguen siendo escasas. Por lo tanto, en el primer caso clínico específico, analizamos un neonato de ballena de Bryde (*Balaenoptera edeni*), que murió poco después del varamiento activo. Esta evaluación se llevó a cabo mediante un examen físico, análisis de sangre, necropsia, histopatología e inmunohistoquímica.

El animal presentó niveles séricos elevados de CK, cTnI, BUN y creatinina. Microscópicamente, observamos espículas de queratina (células epiteliales escamosas) y áreas de atelectasia en los pulmones indicativo de sufrimiento fetal. La degeneración aguda en los

miocitos y cardiomiocitos fue comparable a los hallazgos descritos previamente en casos de miopatía de captura en cetáceos vivos. Se analizaron, además, marcadores de inmunohistoquímica como mioglobina, fibrinógeno y troponina.

Aunque se han documentado daños esqueléticos y miocárdicos en varias especies de cetáceos, sin embargo, este es el primer caso descrito en la literatura de rabdomiólisis esquelética y cardíaca asociada al varamiento activo en una ballena de Bryde recién nacida que sufrió sufrimiento fetal.

Los resultados se publicaron en el artículo titulado ***Skeletal and cardiac rhabdomyolysis in a live-stranded neonatal Bryde's whale with fetal distress***, en la revista *Frontiers in Veterinary Sciences*, Volume 20, Issue 6 (doi: 10.3389/fvets.2019.00476), teniendo esta revista 2.029 de índice de impacto en 2018 y siendo, por eso, una revista Q1 en la categoría de ciencias veterinarias.

ii. Caso clínico específico 2

Las muertes agudas por causas estresantes de los cetáceos varados activamente pueden atribuirse al "síndrome de respuesta al estrés" o "reacción de alarma", que se cree que son comparables a los descritos en la miopatía de captura (CM). La CM consiste en un síndrome metabólico siendo la forma más devastadora de estrés agudo descrita en animales salvajes que puede ocurrir durante y después de la manipulación y transporte de los mismos. Aunque la CM se ha caracterizado en muchas especies de cetáceos, las descripciones del daño cardíaco – un componente importante de este síndrome, y, según autores anteriores, comparables a la patología humana existente, llamada SCMP – aún son escasas (Cowan and Curry 2008; Herráez et al. 2007, 2013). Por lo tanto, en el segundo caso clínico específico, hemos descrito, por primera vez, el análisis bioquímico de muestras de sangre consecutivas *in vivo*, seguidas de las características macroscópicas generales, histopatológicas, histoquímicas e inmunohistoquímicas de la CM, y más específicamente de la SCMP involucrado en este síndrome, causadas por el varamiento activo y el consiguiente intento de rehabilitación, durante un cierto período de tiempo (6 días), en un macho juvenil de calderón gris (*Grampus griseus*).

El animal presentó valores elevados de CK, cTnI y BUN, con algunas variaciones durante el período de rehabilitación.

La cinética de la CK, descrita en la literatura, después de una lesión en el músculo (tanto esquelético como cardíaco) consiste en un aumento en 4-9 horas, alcanzando su punto máximo a las 24 horas, y volviendo a la línea de base 48-72 horas después, a menos que ocurra una nueva lesión o daño permanente. Durante el período de rehabilitación del animal objeto de estudio, hemos conseguido comprobar esta cinética, ya que el animal ha presentado diferentes valores en cada día (día 0 - 837.8 U/L; día 1 - 885.1 U/L; día 2 - 334.1 U/L; día 3 – 959.0 U/L; día 4 - 455.7 U/L; día 5 - 715.3 U/L; día 5 post-eutanasia - 843.6 U/L) (Bonsembiante et al. 2017; Gulland et al. 2018; Lewandrowski et al. 2002; Nachtigall et al. 1990; Seguel et al. 2014). (Lewandrowski et al. 2002). Además, el aumento del valor de este marcador en los días 3 y 5 indica la aparición de una nueva lesión.

La mayoría de los cardiomiocitos lesionados liberan troponina 3-9 horas después del daño isquémico, alcanzando su punto máximo entre 12-48 horas y permanece elevada hasta 4-7 días, a menos que ocurra una nueva lesión o daño permanente (Khan 2015; Lewandrowski et al. 2002). Todos los resultados (día 0 - 0.035 µg/L; día 1 - 0.151 µg/L; día 2 - 0.133 µg/L; día 3 - 0.120 µg/L; día 4 - 0.164 µg/L; día 5 - 0.162 µg/L; día 5 post-eutanasia - 0.168 µg/L) de este animal coinciden con la cinética, excepto los días 4 y 5 (1:30 horas después de la eutanasia), en donde se detecta un nuevo pico atribuible a un nuevo daño.

Histológicamente, detectamos lesiones degenerativas agudas (es decir, necrosis en banda de contracción, mionecrosis que consiste en degeneración fibrilar segmentaria con sarcoplasma eosinofílico hialinizado e hipercontracción, fibras en acordeón, hipereosinofilia y vacuolización citoplasmática) análogas a las observadas en animales con CM (a nivel del músculo esquelético) y en humanos con SCMP (a nivel del músculo cardíaco). Además, también se observó infiltración por células mononucleares a nivel cardíaco. Los riñones mostraron una congestión y se encontró una sustancia amorfa y acidófila dentro de la cápsula de Bowman y en los túbulos renales medulares.

Consideramos que este estudio es una contribución importante a la bioquímica clínica en los cetáceos. El análisis bioquímico de muestras de sangre y, finalmente, la evaluación de la necropsia avanza nuestra comprensión sobre la patología en los cetáceos, ya que podría ayudar en la toma de decisiones y los procedimientos de tratamiento durante los varamientos activos y mejorar los esfuerzos de conservación al reducir la mortalidad de estos animales.

Todos estos resultados se publicaron en el artículo titulado ***Capture Myopathy and Stress Cardiomyopathy in a live-stranded Risso's dolphin (Grampus griseus) in rehabilitation***,

en la revista *Animals,* Volume 10, Issue 2 (doi: 10.3390/ani10020220), teniendo esta revista 1.832 de índice de impacto en 2018 y siendo, por eso, una revista Q1 en la categoría de ciencias veterinarias.

1.4 CONCLUSIONES

PRIMERA. Los cetáceos que mueren durante y/o después de eventos estresantes como los varamientos activos, colisiones con embarcaciones e interacciones accidentales con pesca, desarrollan cambios vasculares y lesiones degenerativo-necróticas agudas cardíacas comparables a las lesiones observadas en humanos con cardiomiopatía por estrés, demostrando así que los cetáceos son susceptibles de sufrir esta patología.

SEGUNDA. El rango de referencia normal para la troponina I cardíaca sérica en delfines mulares adultos nacidos y mantenidos bajo el cuidado humano es de 0 a 0.0256 µg/L. Esta es la primera referencia en la literatura científica de valores sanguíneos para la troponina I cardíaca en cetáceos.

TERCERA. Los cetáceos varados vivos y sometidos a interacción con humanos desarrollan una cardiomiopatía aguda por estrés caracterizada por el incremento sérico de la troponina I cardíaca, que se correlaciona con la depleción tisular de las troponinas cardíacas I y C, en los cardiomiocitos con cambios degenerativos agudos, detectada histológicamente.

CUARTA. Se valida la utilización de la determinación clínica de la troponina I cardíaca sérica y el análisis inmunohistoquímico de las troponinas cardíacas I y C para el diagnóstico y toma de decisiones en la cardiomiopatía de estrés en cetáceos varados vivos.

REGULATIONS & ADAPTATION TO DOCTORAL THESIS BY COMPENDIUM OF PUBLICATIONS

The Regulations for doctoral studies at the University of Las Palmas de Gran Canaria, approved by the Governing Council on 26th February 2019 (Official Bulletin of the ULPGC of 04/03/2019), establishes in article 12 the general requirements that a doctoral thesis must meet for a publication compendium:

1. For the presentation of thesis by compendium of publications it will be necessary:
   e) A minimum of three publications, with a thematic unit, indexed in the *Journal Citations Reports*, *Arts* and *Humanities Citation Index* or equivalent, of which the doctoral student is the first or main author. At least one of them must have been published in a journal whose impact index places it within the first half in decreasing order of impact index among the journals in the area.
   f) In order to accredit the condition of principal author, this should be recognized by the rest of the authors of the publications presented as the nucleus of the doctoral thesis, at the same time that they should renounce using these publications as the principal nucleus of other doctoral theses, without prejudice that these publications can be presented as complementary merits in the doctoral theses that could be presented by the other authors of these publications.
   g) In areas of special technological incidence, two of these publications may be replaced by patents in use or publications in congresses recognized by the ANEP in its scales for obtaining six-year periods.
   h) That the publications or patents include the ULPGC through the affiliation of the director or the doctoral student.
2. Doctoral theses presented as a compendium of publications must comply with the format established in paragraphs 1 to 3 of Article 11 of this Regulation and contain the following paragraphs:
   e) An introduction presenting the objectives of the thesis, the published works and the justification of the thematic unit of the thesis.
   f) A copy of the published work.
   g) The final conclusions.
   h) In the event that the provisions of paragraphs a and c have been written in a language other than Spanish, a summary in Spanish must be included, in accordance with Article 10 of these regulations, of between 5 and 20 pages, including the objectives and conclusions.

This doctoral thesis complies with the provisions of the article mentioned above, as four publications are presented, all of which have the PhD student as the first author and are published in scientific journals within the scope of the doctoral programme. All four publications have the quality marks required by the doctoral programme, i.e. all four articles are indexed in *Journal Citations Reports*.

The following are the indications of quality of the publications:

1. **Nakita Câmara**, Eva Sierra, Carolina Fernández-Maldonado, Antonio Espinosa de los Monteros, Manuel Arbelo, Antonio Fernández, Pedro Herráez. "Stress cardiomyopathy in stranded cetaceans: a histological, histochemical and immunohistochemical study". doi: 10.1136/vr.105562. Veterinary Record. Impact Factor 2018: 2.101. Journal Rank in Veterinary Sciences category: 16/141 (Q1).

2. **Nakita Câmara**, Eva Sierra, Antonio Fernández, Manuel Arbelo, Marisa Andrada, Antonio Espinosa de los Monteros, Pedro Herráez. "Increased plasma cardiac troponin I in live-stranded cetaceans: correlation with pathological findings of acute cardiac injury". doi: 10.1038/s41598-020-58497-3. Scientific Reports. Impact Factor: 4.011. Journal Rank in Multidisciplinary Sciences category: 15/69 (Q1).

3. **Nakita Câmara**, Eva Sierra, Antonio Fernández, Cristian Manuel Suárez-Santana, Raquel Puig-Lozano, Manuel Arbelo, Pedro Herráez. "Skeletal and cardiac rhabdomyolysis in a live-stranded neonatal Bryde's whale with fetal distress". doi: 10.3389/fvets.2019.00476. Frontiers in Veterinary Science. Impact Factor: 2.029. Journal Rank in Veterinary Sciences category: 18/141 (Q1).

4. **Nakita Câmara**, Eva Sierra, Antonio Fernández, Manuel Arbelo, Yara Bernaldo de Quirós, Marina Arregui, Francesco Consoli, Pedro Herráez. "Capture Myopathy and Stress Cardiomyopathy in a Live-Stranded Risso's Dolphin (Grampus griseus) in Rehabilitation". doi: 10.3390/ani10020220. Animals. Impact Factor: 1.832. Journal Rank in Veterinary Sciences category: 29/141 (Q1).

In accordance with the provisions of Article 12 of the Regulation on Doctoral Studies for the presentation of theses by compendium of publications, the thematic unit of the publications contributed is shown. The thematic unit of the publications that integrate this thesis by

compendium is evident, since all of them deal with Stress Cardiomyopathy in cetaceans, having approached this pathology through biochemical, histological, histochemical and immunohistochemical studies.

Finally, the structure of this doctoral thesis complies with the provisions of Article 12.2 of the Regulations on Doctoral Studies, as can be seen in the following pages, as it offers:

d) an introduction presenting the objectives of the thesis, the presentation of the published works and the justification of the thematic unit of the thesis (bibliographic review);
e) a copy of the published works (publications);
f) the final conclusions.

ABSTRACT

Previous studies have shown the susceptibility of cetaceans to stress through blood tests that demonstrate the activation of the hypothalamic-pituitary-adrenal axis with the consequent production and release of glucocorticoids, mineralocorticoids and catecholamines (adrenaline and norepinephrine) (Prosser 1986; Spraker 1993).

Although the beneficial effects of stress system activation are well known, in extreme or prolonged situations, animals can be adversely affected by the effects of physical and psychological stress (Cowan and Curry 2002).

Therefore, marine mammals can develop extreme responses to stress that cause a significant deterioration in animal health and even death. Cardiac lesions seem to play a central role in these adverse responses to stress in cetaceans. Different authors suggest that these animals are particularly predisposed to develop stress cardiomyopathy (SCMP) probably due to the characteristics of their cardiovascular adaptations, conforming to the requirements of diving metabolism (Cowan and Curry 2002; Cowan et al. 2000; Cozzi et al. 2017; Herráez et al. 2007, 2013).

In recent decades, efforts have been made to reduce the impact that some human activities have on free-living cetaceans, but unfortunately whales and dolphins continue to be threatened by: active (live) stranding and interactions with humans, collision with ships and accidental capture (bycatch). These different pathological entities have acute stress, as the centre of its etiopathogenesis, which can cause the death of the animal or seriously aggravate a previous disease situation, while influencing its subsequent rehabilitation, making the therapy and recovery of the animals involved not successful (Arbelo et al. 2013; Bonsembiante et al. 2017; Cowan and Curry 2008; Díaz-Delgado et al. 2018; Duignan and Jones 2005; Gulland et al. 2018; Herráez et al. 2007, 2013; Sierra et al. 2014, 2017; Soulsbury et al. 2008).

The investigation here presented was designed with the aim of continuing the research in cetaceans, with a double intention:

- On the one hand, characterize cardiomyopathy due to stress in cetaceans, trying to provide greater knowledge about their pathologies derived from a situation of acute stress.
- On the other hand, this knowledge would help us find applicable solutions, using health as a necessary instrument for the conservation of cetaceans in our seas.

Thus, based on the above, this will be achieved through specific objectives such as:

1. Characterize SCMP, as injuries resulting from extreme stress responses in live-stranded cetaceans subjected to capture and interaction with humans, as well as in dead cetaceans due to ship strikes and interactions with fisheries (bycatch).
2. Correlate the blood values detected for the biochemical markers of acute cardiac muscle damage with the pathological findings identified histologically, in actively stranded cetaceans and subjected to capture and interaction with humans.
3. Application of the knowledge obtained previously in the diagnosis of specific clinical cases.

This thesis is presented as the compendium of four articles, published in peer-review journals with high scientific impact, describing the results and interpretation of the biochemical, histological, histochemical and immunohistochemical analysis conducted.

This study represents the first biochemical and morphological characterization of SCMP, as an injury resulting from extreme stress response in live-stranded cetaceans and subjected to capture and interaction with humans, dead cetaceans due to collisions with ships and with fishing activities interaction (bycatch).

INTRODUCTION

&

OBJECTIVES

The ocean urges us to contemplate it, overwhelmed by its immensity. However, although we observe it, it is still a liquid blade that conceals another world. Beneath its blue horizon is a saltwater space, a three-dimensional domain full of life. In a medium as uniform as the ocean, there are few boundaries that prevent the passage of species. The currents are authentic freeways through which life circulates freely (Fundación Mapfre Guanarteme 2011).

The stability of the marine environment is a critical condition for the sustainable development of the planet. The protection of marine ecosystems is a global priority. Throughout history, we have accumulated biological, oceanographic, geophysical, etc. data with those who have been able to define risks and propose solutions to maintain a lasting balance between human activities and the conservation of the seas. Among these environmental sciences the study of marine mammals receives a special interest, since, like men, they are homeothermal mammals, with a long period of survival and they are at the top of the trophic chain, constituting excellent bioindicators of marine environment, providing valuable information on the degree of degradation or conservation of this habitat through the biosanitary study of these species.

Consequently, with the advancement of science, the strandings of marine mammals have moved from mere curiosity/fascination throughout history to increasingly detailed studies. It is now important to continue this scientific evolution, which goes through areas as diverse as oceanography, pathology, toxicology, genetics, etc. and to draw as much information as possible on these events in order to know and eventually predict future changes in populations and species. Despite limitations on the stranding study, the record compilation of stranded animals, not only on Canary Islands or Andalusia, but also around the world, provide a rich collection that can be explored alongside the development of other sciences.

Thus, through the study of stranded animals, it is intended to add another drop to the vast sea of scientific knowledge about cetaceans.

Previous studies have shown the susceptibility of cetaceans to stress through blood tests that demonstrate the activation of the Hypothalamic-Pituitary-Adrenal (HHA) axis with the consequent production and release of glucocorticoids, mineralocorticoids and catecholamines (adrenaline and norepinephrine) (Prosser 1986; Spraker 1993).

Although the beneficial effects of the activation of the stress system are well known, in extreme or prolonged situations, animals can be adversely affected by the effects of both physical and psychological stress (Cowan and Curry 2002).

Therefore, marine mammals can develop extreme responses to stress that cause a remarkable deterioration of animals and even their death. In these adverse responses to stress in cetaceans, cardiac lesions seem to play a central role, thus different authors suggest that cetaceans are particularly predisposed to develop Stress Cardiomyopathy (SCMP) probably due to the characteristics of their cardiovascular adaptations conforming to the requirements of diving metabolism (Cowan and Curry 2002; Cowan et al. 2000; Cozzi et al. 2017; Herráez et al. 2007, 2013).

Efforts have been made, in recent decades, to reduce the impact that some human activities have on cetaceans, but unfortunately whales and dolphins continue to be threatened by: active (live) stranding, ship collision and/or fishery interactions such as bycatch. These different pathological entities have the acute stress as the central etiopathogenesis, which can cause the death of the animal or seriously aggravate a previous disease situation, whilst influencing their subsequent rehabilitation, making therapy and recovery of the involved animals unsuccessful (Arbelo et al. 2013; Bonsembiante et al. 2017; Cowan and Curry 2008; Díaz-Delgado et al. 2018; Duignan and Jones 2005; Gulland et al. 2018; Herráez et al. 2007, 2013; Sierra et al. 2014, 2017; Soulsbury et al. 2008).

The research presented, was designed with the general aim of continuing the research in cetaceans with a double intention:

- On the one hand, confirming the hypothesis presented by previous authors, suggesting that cetaceans can develop and suffer from SCMP.
- On the other hand, this knowledge is helping us to find applied solutions, using health as a necessary instrument for the conservation of cetaceans in our seas.

Subsequently, and based on the above, this study was developed with 3 specific objectives, which are:

1. To characterize the SCMP, such as injuries resulting from extreme stress responses in actively stranded cetaceans and subjected to capture and interaction with humans, dead cetaceans due to ship strikes and interactions with fisheries (bycatch).
2. To correlate the blood values detected for the biochemical markers of acute cardiac muscle damage with the pathological findings identified histologically, in actively stranded cetaceans and subjected to capture and interaction with humans.
3. To apply the knowledge obtained previously in the diagnosis of specific clinical cases.

LITERATURE REVIEW

4.1 STRESS

4.1.1 DEFINITION OF STRESS

Nowadays stress is a very commonly used word. It is a part of life and is not intrinsically bad. All forms of life have evolved mechanisms to deal with stressful situations in their lives. In fact, we often look for stress/ "thrill"/ stimulus, and we love its biological stimulant effects, as even being psychologically rewarding. This is why we ski, ride roller coasters and climb mountains, etc. However, nobody denies that stress can have a detrimental effect on the individual, and therefore is seen as a pervasive modern-day killer. We are only very aware of the human diseases associated with a life full of stress, and we are concerned with the effect that stress carries in us. Little by little we have come to accept that animals also bear a great stress load, and that when they suffer from stress they develop very similar pathologies. Like humans, while experiencing severe stress, animals can succumb to the disease, not reproduce or develop properly (Moberg 2000; Yousef 1988). It is the recognition of these negative effects of stress that has sensitized us to the importance of stress for the welfare of an animal. Our challenge is to differentiate between the small non-threatening situations of life and those that negatively affect the welfare of an animal.

To date, animal stress has not been given a universally accepted precise definition. The term stress is very general, and is used quite loosely by various scientists. The divergent definitions are unfortunate, leading to confusion in gathering and integrating available data from literature (Yousef 1988). Stress can be defined as an internal (physiologic or psychogenic) or environmental stimulus that initiates an adaptive change or stress response in an animal (Breazile 1988). Despite stating that, stress is commonly defined as the biological response caused when a person or animal perceives a threat to their homeostasis, being the threat the stressor, it is also not easy to formulate an acceptable definition due to the following (Moberg 2000):

- There are no good biological tests to measure stress;
- Biological responses to stress (behavioural, automatic and neuroendocrine) have a marked degree of interanimal variability;
- Failure to correlate measures of stress and meaningful changes in the well-being of animals.

Stress can be psychological (fear, retention, management or exposure to a new environment) or physical (thirst or hunger, fatigue, trauma or extreme temperatures) (Cowan and Curry 2008).

4.1.2 RESPONSE TO STRESS

a) Stress and catecholamine release

Although the adaptive responses to a stressor, may be different not only depending on the species and/or individual but also because of a complex interplay of genetic factors and previous experiences, it always involves a number of changes including alterations in behaviour, endocrine and autonomic system that induce significant physiological effects on the body through activation of the Hypothalamic-Pituitary-Adrenal axis with the consequent production and release of catecholamines (adrenaline and norepinephrine), glucocorticoids and mineralocorticoids (Gray and Young 1956; Moberg 2000; Prosser 1986; Spraker 1993).

Serum elevation of catecholamines, exogenous or endogenous, is a proven cause of striated and smooth muscle necrosis in humans and animals (Cebelin and Hirsch 1980; Gray and Young 1956; Reichenbach and Benditt 1970).

During the resting state, the sympathetic and parasympathetic nervous system and the adrenal medulla are constantly activated (sympathetic-parasympathetic tone). However, in situations of extreme stress, or when the stressful situation is prolonged in time, a "storm" is triggered. The response induces the activation of the Hypothalamus, which alters the activity of the autonomic nervous system, inhibiting the parasympathetic system and stimulating the sympathetic nerves, resulting in a stimulation of the adrenal medulla and the release of catecholamines and encephalin (Spraker 1993).

The sympathetic nervous system is mainly composed of two molecular chemical signals (catecholamines): epinephrine (adrenaline) and norepinephrine (norepinephrine). Epinephrine is produced and released from the adrenal glands, while norepinephrine is produced and released from nerve axons after stimulation of acetylcholine (Spraker 1993).

Catecholamines produce physiological changes at a general level that prepare the body for physical activity (“fight or flight” response). Some of these effects include an increase in heart

rate, blood pressure, blood glucose levels and a general reaction of the sympathetic nervous system (Spraker 1993).

b) Stress effects in the heart function

Endogenous catecholamines, epinephrine and norepinephrine, are essential neurotransmitters, which in physiological plasma concentrations, exert a positive inotropic action on the heart. However, when administered in large doses, induce myocardial injury, both in animals and in humans (Cebelin and Hirsch 1980; Reichenbach and Benditt 1970; Turnbull and Cowan 1998). Under stress conditions, large amounts of catecholamines are released from the sympathetic nerve endings and the adrenal medulla, and exposure to these high concentrations produces coronary spasm, arrhythmias, contractile dysfunction, myocardial cell damage and extensive necrosis (Dhalla et al. 1992).

4.2 STRESS CARDIOMYOPATHY

4.2.1 HISTORY OF STRESS CARDIOMYOPATHY

Recently, there has been an increasing awareness of an unique cardiac syndrome that has been described as the “Apical Ballooning Syndrome”, “Takotsubo Disease”, and Ampulla or Stress Cardiomyopathy (SCMP), being also referred to as the “Broken Heart Syndrome” in the popular press (Prasad et al. 2008).

The syndrome is characterized by acute but rapidly reversible cardiomyopathy, which consists in a left ventricular systolic dysfunction in the absence of occlusive (atherosclerotic) coronary artery disease (Buja and Butany 2015; Merchant et al. 2008; Prasad et al. 2008).

This distinct cardiac syndrome was originally described in the Japanese population in 1990 and was called “Takotsubo Cardiomyopathy”, named after the octopus trapping pot (Tako, octopus; Tsubo, pot). This distinctive round bottom and a narrow neck, resemble the echocardiographic appearance of the left ventricle during systole in these patients (Figure 1). The echocardiogram during this condition displays transient hypokinesis, akinesis, or dyskinesis in the left ventricular middle segments without apical involvement (Buja and Butany 2015; Prasad et al. 2008).

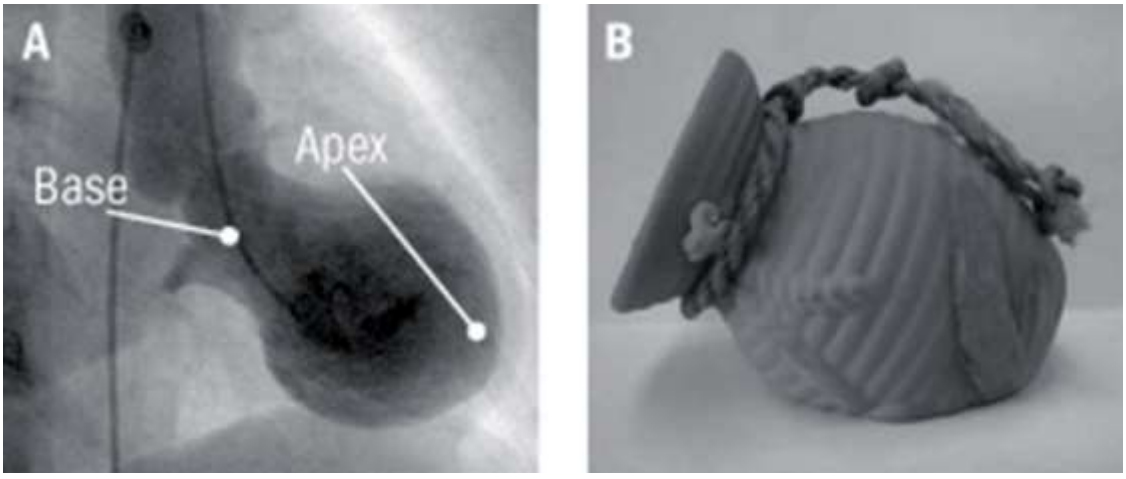


Figure 1. X-ray of the left ventricle (A) shows apical ballooning, a reversible abnormality characteristic of takotsubo cardiomyopathy. During systole (cardiac contraction) the midsection and tip (apex) of the left ventricle balloon out, while the area above, called the base, contracts normally. The shape is similar to that of a tako-tsubo (B), a round-bottomed, narrow-necked vessel used to catch octopuses. Figure

obtained from the website: https://www.health.harvard.edu/heart-health/takotsubo-cardiomyopathy-broken-heart-syndrome.

Nowadays, several cases have been reported from around the world, including different countries from Europe, such as Germany, Belgium, Italy, France, Spain, Portugal or United Kingdom, North America, and Australia (Abdulla et al. 2006; Bybee et al. 2004; Desmet, Adriaenssens, and Dens 2003; Haghi et al. 2007; Huang et al. 1996; Mitchell et al. 2007; Samardhi et al. 2012; Seth et al. 2003; Sharkey et al. 2005; Singh et al. 2014).

4.2.2 CLINICAL SUBTYPES

Stress Cardiomyopathy appears to be related to an excess of plasma catecholamines, which most often occurs after an emotional or physical stress event, and has a clinical presentation that is indistinguishable from a myocardial infarction. Therefore, it is an important differential diagnosis of an acute myocardial infarction, thus it is under recognized and often misdiagnosed (Abdulla et al. 2006; Buja and Butany 2015; Bybee et al. 2004; Desmet et al. 2003; Haghi et al. 2007; Huang et al. 1996; Mitchell and Marquis 2017; Prasad et al. 2008; Samardhi et al. 2012; Seth et al. 2003; Sharkey et al. 2005; Singh et al. 2014).

This syndrome comes to the attention of medical personnel in a variety of clinical scenarios and contexts (Lyon et al. 2016). The different cases can be classified as:

a) *Primary Stress Cardiomyopathy*

The acute cardiac symptoms are the primary reason for seeking medical care. These patients may or may not have a clear identification of the severe, unexpected physical or emotional stressful triggers (being more often emotional). It can also occur spontaneously. Therefore, the potential co-existing medical conditions may be the predisposing risk factors but not the primary cause of the catecholamine rise. The clinical management on these cases depends on the specific complications.

b) Secondary Stress Cardiomyopathy

A substantial proportion of cases occur in patients already hospitalized for another medical condition such as endocrine (e.g. pheochromocytoma), neurological and neurosurgical, respiratory, obstetric, psychiatric, gastrointestinal, infection, babesiosis, cardiological, haematological, surgical, anaesthetic, medication or illicit drugs. In such patients, sudden activation of the sympathetic nervous system or a rise in catecholamines precipitates an acute SCMP as a complication of the primary condition or its treatment. The management in these patients should focus not only on the SCMP and its cardiac complications but also on the condition that triggered the syndrome.

4.2.3 EPIDEMIOLOGY

Stress Cardiomyopathy occurs predominantly in post-menopausal women, being the demographic and clinical characteristics similar in men and women. However, in previous studies demonstrated that emotional stress or anxiety (including in some cases a formal diagnosis of anxiety or panic disorders) or even the absence of identifiable triggers was more common in women. Conversely, a physical stressful triggering event, shock, or resuscitation on presentation was more common in men, who also had higher levels of cardiac biomarkers (troponin). Furthermore, elderly patients are considered to be at higher risk for this syndrome and related major complications, and less of 10% of patients are below 50 years of age (Gianni et al. 2006; Kurisu et al. 2004; Lyon et al. 2016; Ogura et al. 2003; Pilgrim and Wyss 2008; Sharkey 2008).

4.2.4 PATHOPHYSIOLOGY

The pathophysiology is complex and reflects the integrated and systemic physiological responses to acute, severe stress and the cardiovascular responses to sudden surges of endogenous or exogenously administrated plasma catecholamines (Buja and Butany 2015; Lyon et al. 2016).

Catecholamines appear to have a central role in the pathophysiology of this syndrome, as the trigger is often a sudden, unexpected stress. It is necessary to take in consideration two initial elements of the physiology: the first being how much epinephrine and norepinephrine is

released from the hypothalamic-pituitary-adrenal axis in response to a given stress; and the second is the response of the cardiovascular system (including the myocardium, coronary arteries and peripheral vasculature) and the sympathetic nervous system to the sudden sympathetic activation and surge in circulating catecholamines (Helen et al. 2012; Lyon et al. 2016; Pelliccia et al. 2018; Suzuki et al. 2014).

Several hypotheses have been proposed to explain the unique cardiac appearance in this syndrome and the cardiac response to severe stress. These hypotheses can be broadly divided into vascular and/or myocardial causes and may not be mutually exclusive, as the entire cardiovascular system is exposed to the same catecholamine storm. The vascular abnormalities consist in acute multivessel coronary spasm, aborted myocardial infarction with spontaneous recanalization, and acute increased ventricular afterload. Acute left ventricular outflow tract obstruction and direct catecholamine-mediated myocardial stunning are the myocardial irregularities, and finally, the vascular and myocardial motive is the integrated cardiovascular physiology (a cardio-circulatory syndrome) (Angelini 2008; Camici and Filippo 2015; Crea, Camici, and Bairey Merz 2013; Lyon et al. 2016; Naegele et al. 2016; Vitale, Mendelsohn, and Rosano 2009).

Therefore, new information has supported the concept that this pathology is caused by acute myocardial ischemia (Jaffe et al. 2018; Pelliccia et al. 2018).

The ischemia-reperfusion injury is a phenomenon described as an accelerated injury to the heart due to the resupplied blood to the ischemic area of the heart which was previously deprived of its blood supply (ischemia) and suffered from hypoxia, substantial loss of cell volume regulation and influx of calcium because of inadequate run of the ion pumps. Added membrane damage is a result when blood supply is re-established to areas with potentially viable cells (Buja 2005; Buja and Butany 2015).

The crucial role of myocardial ischemia in Takotsubo syndrome, has emerged thanks to the evidence that most of these cases occur in patients with risk factor for endothelial dysfunction which is characterized by an imbalance between vasoconstricting and vasodilating factors that could explain the propensity to epicardial and/or microvascular coronary artery spasm of the disease. This pathologic state of the endothelium may represent an important link between stress and myocardial dysfunction in SCMP. There is now an agreement that the increased concentration of catecholamine occurring in the acute phase of SCMP contributes to an acute situation of “supply-demand mismatch” followed by post-ischemic stunning, since it

induces direct myocardial dysfunction in sarcoplasmic reticulum calcium channels, leading to leakage of calcium into the sarcoplasm, contraction impairment, myocardial necrosis and coronary vasoconstriction, mostly at the microvascular level, together with an increased cardiac workload (Pelliccia et al. 2018; Seguel et al. 2014).

4.2.5 CLINICAL PRESENTATION AND DIAGNOSIS

Patients with SCMP typically present with acute chest pain of cardiac origin (angina), breathlessness, and palpitations due to sinus tachycardia or arrhythmia. In more severe cases, pre-syncope or syncope due to ventricular tachyarrhythmia's, severe left ventricular outflow tract obstruction, or cardiogenic shock may be present. They may describe a wave of pressure from the chest to the neck and into the head, consistent with the acute catecholamine and hypertensive surge and frequently associated with diaphoresis and heightened anxiety.

The diagnostic criteria for SCMP established by the Heart Failure Association consists in alterations in medical exams, such as electrocardiography (ECG), coronary angiography and left ventriculography, echocardiography, cardiac magnetic resonance, coronary computed tomography angiography, and biochemical analysis (Lyon et al. 2016).

a) Electrocardiography (ECG)

More than 95% of the SCMP patients present irregularities on ECG during the acute phase (under 12 h). These new and reversible abnormalities consist in ST-segment elevation, ST depression, new left bundle brunch block (which may be permanent after the syndrome, but should also alert clinicians to exclude other cardiomyopathies), and sometimes Q-waves, with deep and widespread T-wave inversion, and/or QT prolongation developing 24-48 h after the onset of symptoms or the precipitating stressful trigger when present (which can take many weeks to months to normalize after recovery of left ventricular function) (Haghi et al. 2007; Kurisu et al. 2004; Lyon et al. 2016; Mitchell et al. 2007; Ogura et al. 2003; Sharkey 2008).

b) Coronary Angiography and Left Ventriculography

The imaging exams, mainly coronary angiography and left ventriculography, reveal that patients with SCMP present transient regional wall motion abnormalities of the left ventricle or

right ventricle myocardium which are frequently, but not always, preceded by a stressful trigger (emotional or physical). These irregularities usually extend beyond a single epicardial vascular distribution, and often result in circumferential dysfunction of the ventricular segments involved. It is also necessary to observe the absence of culprit atherosclerotic coronary artery disease including acute plaque rupture, thrombus formation, and coronary dissection or other pathological conditions to explain the pattern of temporary left ventricle dysfunction detected (e.g. hypertrophic cardiomyopathy, viral myocarditis) (Abdulla et al. 2006; Desmet et al. 2003; Haghi et al. 2007; Lyon et al. 2016; Mitchell et al. 2007; Prasad et al. 2008; Sharkey et al. 2005).

c) Biochemical Analysis

Reference values, both haematological and biochemical, are vital to provide baseline, screening, and diagnosis of diseases. Healthy subjects are able to maintain the biochemical concentration of certain molecules in each cell or biological fluid within the limits of concentrations considered normal or usual values. For that reason, variations of these biological parameters, both qualitative and quantitative, may indicate a pathological state. Consequently, the assess of a pathological condition through the measurement of the degree of a modification of a biochemical parameter is one of the very aims of clinical biochemistry (Kamdje, Nkem, and Mimfoumou 2017). Considering this, the biomarkers, cardiac troponin I (cTnI) and/or creatine kinase (CK), have a small, rapid increased to above normal levels, the brain natriuretic peptide (BNP) may be elevated, and plasma catecholamines may be also elevated (Fineschi et al. 2010; J. et al. 2008; Lyon et al. 2016).

i. Creatine Kinase (CK)

The most commonly used serum enzyme in the determination of neuromuscular diseases of domestic animals is creatine kinase (CK), previously designated by creatine phosphokinase (Valberg 2008).

There are three main forms of CK isoenzymes. Creatine kinase has a dimeric structure consisting of M (muscular) subunits and B (cerebral) subunits, which combine to form the three heterogeneous isoenzymes: heterogeneous MM (or CK3), MB (or CK2) and BB (or CK1) (Valberg 2008). In humans, CK-MB is present in a relatively high concentration in the myocardium (approximately 20% of the total myocardial CK), while the concentration of CK-MM is the highest

in skeletal muscle (98% of the total muscle CK), with only a small amount of CK-MB (usually about 2%) (Lewandrowski et al. 2002).

Creatine kinase rises in less than 3 to 12 h after the injury, with peaks within 12 to 24 h and returns to the baseline after 48 to 72 h, unless a new injury or permanent damage occurs (Lewandrowski et al. 2002).

Although CK is considered a sensitive marker of myocardial damage, it is also present in skeletal muscle in high concentrations as well as in the intestine, diaphragm, uterus, and prostate in minor amounts, thusly it has poor specificity to detect heart damage (Khan 2015).

ii. Troponin

Consequently, troponins have been adopted, over other cardiac markers, such as CK, as the new gold standard for necrosis, since cTnI is detectable in very low amounts (for example 0.01 µg/L) in blood from healthy individuals with no evidence of cardiac disease (Hassan et al. 2009; Lewandrowski et al. 2002; Morrow et al. 2007; Venge et al. 2009). Therefore, it is thought that significant elevations (≥0.1 µg/L) of this marker is most likely reflecting myocardial necrosis. When irreversible myocardial damage occurs, troponin is released from cardiomyocytes. This is a regulatory complex protein comprising three subunits, which are Troponin C (calcium binding component), Troponin T (binding component tropomyosin) and Troponin I (inhibiting component). All of these are involved in the contractile process of both skeletal and cardiac muscle. The Troponin C expressed in the muscle is identical to the cardiac troponin C (cTnC). However, cardiac troponin T (cTnT) and cardiac troponin I (cTnI) are specific to the heart (Coudrey 1998). Due to this specificity, cTnT and cTnI are currently recommended by various international societies as a diagnostic indicator for acute myocardial infarction since they have the potential to be specific markers of cardiac damage (Morrow et al. 2007). Thus, as mentioned previously cardiac troponin is released from cardiomyocytes into the blood in proportion to the degree of damage, as a result from an injury, consistent with ischemia or various other causes (Hassan et al. 2009).

Injured cardiac myocytes release troponin, for the most part, 3 to 9 h after ischemic damage, reaches its peak 12 to 48 h and its elevation stay rose for up for 4 to 7 days in case of cTnI (Hansen and Rossen 1999; Khan 2015; Lewandrowski et al. 2002). Early recognition of myocardial necrosis (1 to 3 h) is not allowed by the view of this kinetics, and these markers do not help maximal affectability until 6 or more hours after the onset of the acute myocardial

infarction and/or SCMP. Since precise determination of the timing of stress event and/or symptom onset is frequently clinically exceptionally difficult because it is focused around patient report. Therefore, a precondition to get a satisfactory clinical affectability to distinguish these pathologies in humans is that the blood for testing should be collected at 6 to 9 h after onset of manifestation (Khan 2015). Moreover, previous studies propose that the hence measuring troponin in serum can be an important auxiliary method in examining sudden death seeing that its peak concentration can be related with the degree of injury (Khan 2015).

More than 90% of patients with SCMP revealed raised cTnI when measured by conventional assays (Lyon et al. 2016). However, SCMP is under-recognized because of the unnecessary delay in the diagnosis process in postmortem examination cases of acute myocardial infarction caused by the less sensitive conventional markers. Therefore, it is necessary to establish diagnostic utility of different biochemical cardiac markers in biological fluids for postmortem diagnosis of acute myocardial infarction and/or SCMP due to the limitations of histopathological findings. Thus, the detection of even microinfarction and acute myocardial infarction much earlier after the onset of ischemia by using a rapid one-step assay in body fluids in autopsy cases is allowed by the sensitivity of cTnI (Khan 2015).

iii. Brain Natriuretic Peptide (BNP)

Type B natriuretic peptide (BNP) has shown promise as a new diagnostic marker for congestive heart failure. The level of plasma BNP has been shown to correlate with long-term cardiovascular mortality in human patients with acute myocardial infarction and is an independent predictor of left ventricular ejection fraction and heart failure in these patients. The level of BNP correlates with the amount of ventricular dysfunction present after an acute myocardial infarction and may, in the future, become an useful screening test to identify patients with a higher risk of morbidity and mortality (Lewandrowski et al. 2002). This marker increases rapidly within 6 h, reaching its peak usually by 24-48 h and at 1 month levels are similar to baseline (Agarwal et al. 2015).

4.2.6 HISTOPATHOLOGY

The histological findings, both in human myocardial samples and in experimental models of SCMP, are consistent with catecholamine cardiotoxicity, due to the ischemia-reperfusion

injury (Akashi et al. 2010; Buja 2005; Buja and Butany 2015; Fineschi et al. 2010; Jaspreet et al. 2019; Jiang and Downing 1990; Kawai 2012; Maréchaux et al. 2008; Mitchell and Marquis 2017; Miura et al. 2017; Pascual et al. 2015; Prasad et al. 2008).

From several histological analysis of myocardial tissue obtained through, biopsied and postmortem examined specimens, it is known that SCMP is associated with different vascular changes essentially congestion, haemorrhages or interstitial edema. As well as severe morphological alterations, consisting of acute degenerative changes, such as the presence of contraction band necrosis, hypereosinophilia, and many vacuoles of different sizes contributing to the cellular deterioration. Not to mention infiltration by mononuclear lymphocytes, leukocytes and macrophages (Akashi et al. 2010; Fineschi et al. 2010; Jaspreet et al. 2019; Jiang and Downing 1990; Kawai 2012; Maréchaux et al. 2008; Mitchell and Marquis 2017; Miura et al. 2017; Pascual et al. 2015; Prasad et al. 2008).

Subendocardial congestion, fibers separated by spaces representing interstitial edema, and haemorrhages are early histological findings included in the pathology of a catecholamine cardiomyopathy (Jiang and Downing 1990).

Vascular congestion is the engorgement of a vascular bed generally caused by a decreased outflow with a normal or increased inflow of blood (Fishbein 2001; Jaspreet et al. 2019; Mosier 2017).

Following administration of catecholamines, interstitial edema (represented by spaces in the fibers which are separated) is usually associated with subendocardial and subepicardial haemorrhages and is characteristically present in damaged areas of the myocardium, even after 72 h (Fishbein 2001; Jaspreet et al. 2019).

Focal and diffuse subendocardial haemorrhages are visible on gross examination, being also noted sometimes in the myocardium and epicardium, shortly after large doses of catecholamines are given and/or released, in humans with SCMP (Jiang and Downing 1990; Mitchell and Marquis 2017).

Contraction band necrosis is described as focal hypercontraction and lysis of contractile filaments in small groups of myocardial cells. The resulting dense hypereosinophilic bands run transversely through the myocyte. It develops after transient ischemia and reperfusion, is visible within 5 to 10 minutes, and is the characteristic skeletal and myocardial injury associated with the administration of catecholamines or with high concentrations of endogenous

catecholamines (Fineschi et al. 2010; Reichenbach and Benditt 1970; Turnbull and Cowan 1998). Therefore, is a lesion widely described as indicator of stress cardiomyopathy (Akashi et al. 2010; Fineschi et al. 2010; J. et al. 2008; Jiang and Downing 1990; Kawai 2012; Maréchaux et al. 2008; Mitchell and Marquis 2017; Miura et al. 2017; Pascual et al. 2015; Prasad et al. 2008),

The first histologic abnormality associated with ischemia is the presence of long and thinned undulated fibers, denominated as wavy fibers (Fineschi et al. 2010; Fishbein 2001). The wavy fibers are likely to result from stretched fibers that cannot contract, as does the adjacent myocardium, during bulging of severely ischemic tissue that occurs during systole. This finding serves as a further criterion for the morphological recognition of early myocardial injury, however, wavy fibers are not specific for necrosis, since they have been observed experimentally shortly after coronary occlusion in the myocardium (Fishbein 2001).

Hypereosinophilia is the first confirmed change specific for myocardial necrosis using histological and histochemical techniques, and consists in an increased eosinophilic staining of necrotic cardiomyocytes (Fineschi et al. 2010; Fishbein 2001). In experimental animals, this colour change and subtle interstitial edema is evident 2 to 3 h after coronary occlusion and will be more pronounced and more easily recognizable from 3 to 6 h (Fishbein 2001). Hypereosinophilia is more pronounced in the subendocardial and subepicardial zone, being supported by previous studies which shows that irreversible injury progresses in a wavefront movement from the ischemic subendocardium, where the perfusion deficit is most severe into the subepicardium, which receives some collateral blood flow (Buja 2005; Cowan and Curry 2002, 2008).

In the literature, vacuolar degeneration is characterized, morphologically, by intracellular accumulation of fluid and lysis of myofibrils. The increase of liquid exerts pressure on the different constituents of the cell, and because of this most of the fibers with vacuoles have a pyknotic nucleus. It is commonly seen in the periphery of myocardial infarctions, and in the subepicardial, subendocardial and perivascular regions which suffer with severe, chronic and fatal ischemia (Adegboyega et al. 1996). Although its occurrence is quite common, the morphological characteristics and the functional importance of the vacuolar degeneration has not generally been appreciated and even though pathogenesis is not known, it has been hypothesized that it may be the result of increased hypoxia induced in myocardial cell membrane permeability with consequent fluid influx which is normally observed in acute deaths associated with stressful situations (Adegboyega et al. 1996).

Another anatomopathological finding, described in postmortem examination and endomyocardial biopsies of human patients and experimental animals who suffer from SCMP, is interstitial infiltration of lymphocytes, leukocytes and macrophages (Jaspreet et al. 2019; Mitchell and Marquis 2017; Pascual et al. 2015).

A well-characterized sequence of events follows the onset of myocardial necrosis and allows for understanding of the progression of changes following onset of acute ischemic injury as well as identification of features useful in determining the age of the lesion. Therefore, in the next table it is possible to observe the chronology of the light microscopic changes described previously (Buja and Butany 2015).

Chronology of the changes	Starting time	Light microscopic changes
Acute	5 minutes	Myocardium with long, thinned, wavy fibers separated by spaces representing edema, and microvascular congestion, at borders of ischemic myocardium.
	15 to 20 minutes	Cell death may starts to develop.
	90 minutes	Fine changes in the sarcoplasm of some myocardial fibers.
	2 to 3 h	Early changes of cardiomyocyte coagulation necrosis with nuclear pyknosis, colour change, more specifically “brick red change” or cytoplasm hypereosinophilia, focal contraction bands and subtle interstitial edema are evident.
	3 to 6 h	Hypereosinophilia and edema become more pronounced and more easily recognizable.
	6 to 12 h	Increased number of neutrophils line up in capillaries, so-called margination of neutrophils. Changes accelerated and more extensive contraction band necrosis with reperfusion
	12 to 24 h	Extravasation into interstitial space of neutrophils. Vascular congestion, interstitial edema, and focal areas of haemorrhage are also recognized. Continuing coagulation necrosis with karyorrhexis and karyolysis of nuclei. Thinning of myocytes in central region and contraction band necrosis toward periphery.
Subacute	1 to 3 days	Heavy influx of neutrophils in periphery. Advanced coagulation necrosis with loss of nuclei and striations. Lymphocytes start to appear at the edge of the infarction. Pigmented mononuclear cells containing lipofuscin appear. Early degeneration of neutrophils.
	3 to 7 days	Degeneration of neutrophils becomes clearly evident producing basophilic granular debris referred to as “nuclear dust”. Beginning of disintegration of necrotic myocytes with phagocytosis and removal of necrotic fibers. Lymphocytes presence is evident. Influx of macrophages; Onset of vascular and connective (granulation) tissue proliferation and early fibrosis has begun at edge of the injury.

Chronology of the changes	Starting time	Light microscopic changes
Chronic	2 weeks	Chronic inflammation and removal of peripheral muscle fibers occur. Increased numbers of mononuclear cells (macrophages) are apparent at the margins of the injury where vascular and connective tissue proliferation has become more prominent. Eosinophils, lymphocytes and plasma cells are also present. Infrequent presence of neutrophils.
	3 to 4 weeks	Continued removal of necrotic fibers and proliferation of granulation tissue extends throughout the injured area. Macrophages are still numerous, and plasma cells, eosinophils and lymphocytes are still present, but there is more substantial fibroblastic proliferation with collagen formation recognizable as pink fibrillary extracellular material.
	5 to 6 weeks	Removal of residual necrotic fibers and continued gradual scar formation with deposition of collagen. Inflammatory cells and vascularity decreased, but a few macrophages, plasma cells, and lymphocytes persist and remain in the scar that eventually forms.
	After 3 months	Fibrous scar is fully developed in most infarcts

4.2.7 IMMUNOHISTOPATHOLOGY

The histological findings are corroborated with specific markers which better expose a myocardial damage. Therefore, the main markers that have been used in studies of human heart samples with SCMP are for example cTnI, cTnC, myoglobin and fibrinogen (Fishbein et al. 2003; Hansen and Rossen 1999; Martínez-Díaz et al. 2005; Ortmann et al. 2000; Xiaohong et al. 2002).

The leakage of muscle (skeletal and cardiac) proteins begins immediately after vital trauma, which causes an acute ischemia, producing early myocardial cell membrane rupture, causing a quickly decline of myoglobin, cTnI and cTnC content and deposition of plasma proteins such as fibrinogen in cardiomyocytes. Homogenous labelling for myoglobin is demonstrated in normal cardiac muscle while injured cardiomyocytes shows both depletion of myoglobin within muscle fibres and intercellular and interstitial accumulation of myoglobin (Ortmann et al. 2000; Xiaohong et al. 2002).

In cells undergoing apoptosis there is a greater concentration of troponin due to condensation, while in zones of obvious ischemic necrosis (infarction zones) tissue antigens are

severely depleted (Fishbein et al. 2003; Hansen and Rossen 1999; Martínez-Díaz et al. 2005; Ortmann et al. 2000).

4.3 CETACEANS

4.3.1 ORDER CETARTIODACTYLA

SUBORDER CETANCODONTA

INFRAORDER CETACEA

The previous *Suborder* and actual *Infraorder Cetacea* (from Latin: cetus meaning whale and from Greek: ketos meaning sea monster) is represented by a diverse group of mammals (whales, porpoises and dolphins) fully adapted to aquatic life, whose specialized anatomy and behaviour mask their terrestrial origin (Motta 2006; Perrin 2020; Perrin, Würsig, and Thewissen 2009).

Therefore, these exclusively aquatic animals that live mostly in the seas, and can also be found in rivers and estuaries, are recognized as mammals for having their own characteristics of this group. Among them can be mentioned the presence of mammary glands, all are endothermic and their breathing is aerial through the lungs.

Taxonomy is constantly changing and the taxonomy for cetaceans is no exception. According to Perrin, Würsig, & Thewissen (2009), cetaceans were divided into two living infraorders (*Mysticeti* and *Odontoceti*) and one extinct infraorder (*Archaeoceti*). Nowadays, and as stated in the World Register of Marine Species by Perrin (2020), these two living infraorders are defined in two superfamilies:

a) Superfamily Mysticeti

The superfamily Mysticeti is composed of mysticetes, represented by 14 species of toothless whales, grouped into 4 families: Balaenidae, Neobalaenidae, Eschrichtiidae and Balaenopteridae. The Balaenopteridae is most variable, with 8 species in two genera (*Balaenoptera* with 7 species and *Megaptera* with only one). This superfamily has as its main features a symmetrical skull, the presence of fins, two breathing holes and buccal beards, which are structures similar to sieves located at the top of the mouth and made of keratin. Mysticetes use beards to filter out plankton from water, small crustaceans and some small fish species (REMANE 2005). Usually males are smaller than females; It has solitary behaviour, except in the

breeding and feeding areas. Generally speaking, species of Balaenoptera vary in size, distribution and behaviour, but species boundaries may be blurred. Species are distinguished mainly on aspects of the feeding apparatus baleen size and spacing, size and shape of the upper jaw, and skeletal differences are rather minor. Thus disparity appears low. The gray whale, *Eschrichtius robustus*, is structurally quite different (disparate) from other mysticetes and is generally placed in its own monotypic family, although some molecular studies place it within the Balaenopteridae.

b) Superfamily Odontoceti

The superfamily Odontoceti encompasses the largest number of species, some of which are fluvial. The navies may be coastal, oceanic or may appear along the continental shelf. The odontocetes are composed of at least 74 species in 10 families of dolphins in general, orcas, all porpoises, sperm whales (the largest representative of this infraorder) and “beak whales” (REMANE 2005), which are characterized by the presence of a breathing hole and teeth (ranging in number from 2 to 200). Usually the teeth are all the same and there is only one dentition, meaning there is no replacement for lifelong missing teeth. A remarkable ability of this group is to locate their prey (fish of various sizes and cephalopods, and may, in the case of orca, devour animals such as seals and penguins) by echolocation. Unlike mysticetes, the skull of odontocetes is asymmetrical. In some species the frontal region and the face are quite developed, in others the mouth is extended forward forming a kind of long sharp beak. Males are generally larger than females, live in groups and their length can range from 1.5 meters to 18 meters in length. Delphinidae is the most diverse family of cetaceans, and disparity seems much higher than, e.g., within Balaenopteridae. Among beaked whales (Ziphiidae; 21 species), the genus *Mesoplodon* has 14 rather similar species in which only adult males are separated easily. Disparity here appears low and awaits explanation in terms of evolutionary ecology within the genus. Among other odontocetes, 4 species of small long-beaked “river dolphins” each represent a single family: Iniidae, Pontoporiidae, Lipotidae, and Platanistidae. Although these “river dolphins” are superficially similar externally, they differ markedly in skull form.

4.3.2 BIOINDICATOR OF THE HEALTH OF MARINE ECOSYSTEMS

Cetaceans are the mammals best adapted to the aquatic environment. They evolved from terrestrial mammals between 55 and 34 million years ago. Although their history is closely related to man, as he has hunted them since time immemorial, the mystery still surrounds these animals. The competition for space led them to dominate the pelagic environment, to specialize in certain fishing resources and to reach depths of 2,000 meters in search of their prey (Fundación Mapfre Guanarteme 2011).

Plankton is the largest settler in the pelagic space and lives freely in the ocean's water column, often being swept away by ocean currents. For example, in order for a pilot whale to reach one ton of weight, it must first eat five thousand tons of plankton. Thus, plankton is at the base of the food chain of aquatic ecosystems as it feeds larger organisms such as cetaceans, which are at the highest end of the food chain (Fundación Mapfre Guanarteme 2011).

And as such, cetaceans are an excellent bioindicator of the state of the marine environment, currently constituting one of the marine mammal groups on which much of the social and scientific interest is centred.

All the knowledge gained from these animals will allow us to work with scientific grounds on their conservation objectives and to detect problems that can directly or indirectly affect marine ecosystems and at the same time animal and sometimes human health.

Because they are wild animals, which encompass numerous species, with an important variety of behaviours, migratory routes, feeding, reproductive cycles, etc. there are numerous and deep gaps in the knowledge of these animals, from the biological point of view as health.

4.3.3 STRESS IN CETACEANS

Physiological stress in cetaceans occurs in the necessary adjustments to adapt to diving, to escape from predators or inter and intraspecific aggression, and catch prey, or even occur when a wild animal is restricted or locked (Cowan and Curry 2008).

So it is clear that the question is not whether dolphins are "under stress" in a given condition, since they are always under the demand for adaptation, in a constant condition of adjustment of physiological systems to maintain homeostasis, but if the degree of stress experienced is physiologically harmful (Cowan and Curry 2008).

a) Response to stress in cetaceans

Marine mammals, in this case cetaceans, present the same basic response to stress as humans and other mammals (Curry 1999). Once the central nervous system perceives a threat, a biological or defence response is developed (Cowan and Curry 2002). This response is well documented in marine mammals with several studies, and includes an adrenocortical response and effects on thyroid hormonal balance (St. Aubin and Geraci 1988; St Aubin and Geraci 1992).

Cortisol levels have been elevated in cetaceans exposed to stress situations, such as capture, handling and retention, although this elevation seems to be modest if we compare it with that experienced by other mammals in similar stress situations. In contrast, aldosterone is significantly increased compared to adrenocortical stimulation in the case of marine mammals (cetaceans and pinniped) (St. Aubin and Geraci 1986; Fair and Becker 2000; Thomson and Geraci 2011).

As previously mentioned, although physiological stress has some benefits, an extreme or prolonged response with release of catecholamines, high blood cortisol and aldosterone levels are potentially damaging to skeletal and heart muscle (Cowan and Curry 2002).

4.3.4 PATHOLOGICAL ENTITIES / CAUSES OF DEATH IN CETACEANS ASSOCIATED WITH STRESS

Free-living cetaceans can be threatened, daily, by a wide variety of stressful situations, of natural and anthropic origin, that affect their well-being.

Different pathological entities have the central axis of their pathogenesis to acute stress. Among these are active stranding and interaction with humans (capture myopathy), but also others such as collisions with boats or fishing activities (bycatch) (Arbelo et al. 2013; Díaz-Delgado et al. 2018; Herráez et al. 2007, 2013; Maldonado 2015; Sierra et al. 2014).

All these pathological entities share clinico-lesional findings, where, among others, degenerative cardiac, acute or sub-acute necrotic lesions, may cause the death of the animals themselves or seriously aggravate a previous disease situation at that time, and it can condition its subsequent rehabilitation, making therapy unsuccessful as well as subsequent recovery (Adegboyega et al. 1996; Arbelo et al. 2013; Cowan and Curry 2002, 2008; Díaz-Delgado et al. 2018; Duignan and Jones 2005; Herráez et al. 2007, 2013; Seguel et al. 2014; Sierra et al. 2017, 2014; Soulsbury et al. 2008).

a) Active/Live Stranding

Active stranding is an unnatural situation in all cetaceans, defined as the one in which the cetacean is alive on the beach or in shallow water in distress due to being unable to free itself and resume natural activity (Geraci and Lounsbury 2005; Gulland et al. 2018; Wilkinson 1991). Regardless of the prior health status of the animals, the stranding implies an anomalous and extreme situation for an organism that is not anatomically and physiologically adapted to environmental conditions different from those of the aquatic environment.

It is a pathological entity "per se" in which acute stress is the central axis of its etiopathogeny presenting clinical-lesional findings that can cause the death of the animal or seriously aggravate a previous disease situation over the period of capture or captivity, which may influence the subsequent rehabilitation and recovery of affected animals, since live stranded cetaceans are frequently debilitated when rescued. Therefore, although the stranding response is oriented to improve the health and the welfare of these animals, some “rescue and recovery” activities that seem logical to us, the interaction with humans, transport and rehabilitation, may in fact be counterproductive (Arbelo et al. 2013; Bonsembiante et al. 2017; Cowan and Curry 2008; Díaz-Delgado et al. 2018; Duignan and Jones 2005; Gulland et al. 2018; Herráez et al. 2007, 2013; Sierra et al. 2014, 2017; Soulsbury et al. 2008).

In cetaceans, acute and stressful deaths, such the ones that occur in live-stranding, may be attributed to the so called “stress response syndrome” or “alarm reaction”, which consists in hyperthermia, catecholaminergic and neurogenic shock, and also assigned to the multiorgan failure, e.g., hemodynamic shock and renal dysfunction. These mechanisms are thought to be analogous to *Capture Myopathy*, in which stress cardiomyopathies seem to have an important role, since it is the most devastating form of acute stress described in birds, terrestrial and marine wild mammals, for instance sea otters, seals, and cetaceans, that may occur during and

after the stress of capture, handling, restraint, and transport of the animals (Cowan and Curry 2008; Díaz-Delgado et al. 2018; Geraci and Lounsbury 2005; Herráez et al. 2007, 2013; Seguel et al. 2014).

The etiopathogenesis in these species are not fully resolved despite the high incidence of active stranding-associated lesions. The stress response of this syndrome resides in different pathological findings that varies and include biochemical changes consistent with elevated serum muscle enzyme activities, specifically CK, which represents variable degrees of myopathy, and, in ataxic myoglobinuric syndrome, elevated serum urea associated with myoglobinuria. In relation to the resulting damage on the marine mammals' heart, more specifically in cetaceans, until now there are no studies where specific cardiac markers have been analysed. Local to generalized vasospasm and vasodilation (catecholamines, neurogenic shock, impeded venous flow return by body compression), direct traumatic injury of muscle and viscera, and reperfusion damage, are the major pathological mechanisms associated with the deaths due to the live stranding (Akashi et al. 2010; Bonsembiante et al. 2017; Buja 2005; Buja and Butany 2015; Cebelin and Hirsch 1980; Dhalla et al. 1992; Díaz-Delgado et al. 2018; Fineschi et al. 2010; Herráez et al. 2007, 2013; Jaspreet et al. 2019; Jiang and Downing 1990; Kawai 2012; Maréchaux et al. 2008; Mitchell and Marquis 2017; Miura et al. 2017; Pascual et al. 2015; Prasad et al. 2008; Reichenbach and Benditt 1970; Seguel et al. 2014; Spraker 1993; Turnbull and Cowan 1998).

b) Ship Collision

The collision of vessels with cetaceans is a recognized and important global threat with a rapid increase in its incidence due to the development of maritime traffic on a global scale, fleet size, and the increase in speed (more than 35 knots). The most serious lethal injuries are caused by large vessels and by those traveling at speeds exceeding 14 knots. This entity contemplates those individuals who have suffered severe trauma after impact with a boat and generally death occurs in an acute form, derived from haemorrhagic shock (hypovolemic), involvement of vital organs directly (e.g., section of the spinal cord), or chronically, by development of sepsis or generalized weakness (Arbelo et al. 2013; Díaz-Delgado et al. 2018; Sierra et al. 2017).

More recently, acute stress has been proposed to have a central role in the over-acute death of animals that suffer collisions with boats, where both skeletal degenerative-necrotic

lesions are observed (in muscles other than traumatized), but also cardiac attributable to the massive discharge of catecholamines (Sierra et al. 2014).

c) Bycatch

The fishing gears that pose the greatest danger to cetaceans include: gill nets, trawls, trammel nets, purse seines and longlines (RR, McClellan, and B 2013). Because of their low cost and widespread use, gillnets are responsible for a large proportion of the incidental catches of cetaceans worldwide. When trapped in fishing nets, small whales, dolphins and porpoises die often because they are not strong enough to break free or surface to breathe. Large whales can usually be released, however, they can continue to be entangled for long periods, causing debilitating injuries and even slow death (Moore et al. 2013).

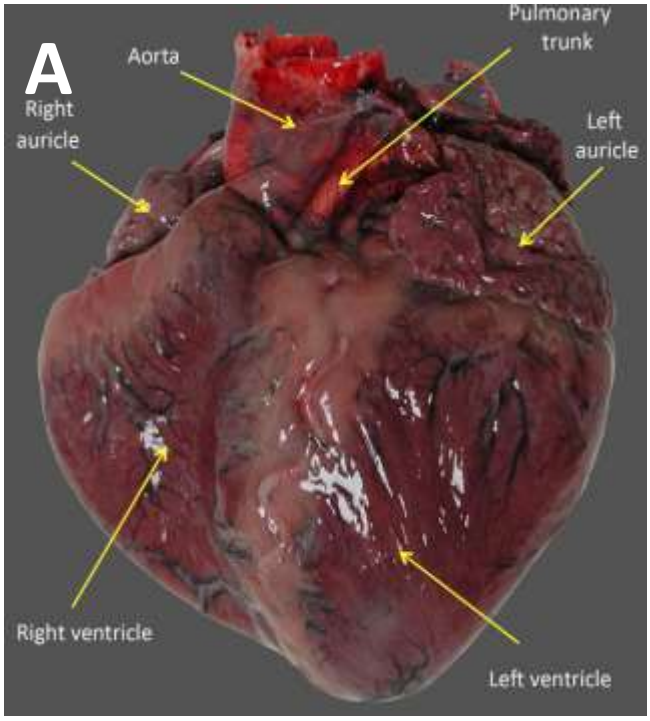
There is a series of agreed criteria in the diagnosis of accidental capture in cetaceans related to health status, contact with fishing gear, the release of the net and related to the lack of oxygen (hypoxia). In captured cetaceans, typical hemodynamic changes of shock, due to the submersion or drowning (asphyxia) are recognized, with macroscopic or histopathological lesions evident at the cardiovascular and respiratory levels (Arbelo et al. 2013; Díaz-Delgado et al. 2018; Duignan and Jones 2005; Soulsbury et al. 2008).

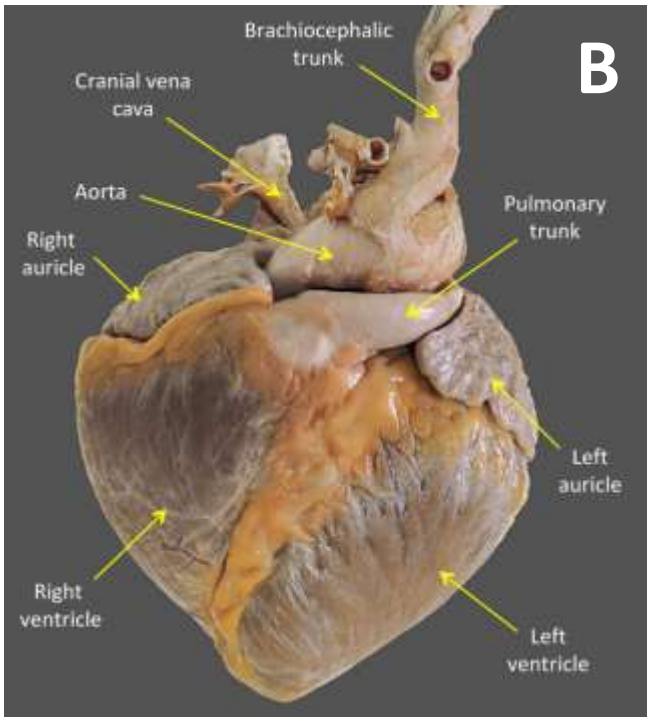
4.3.5 THE HEART OF CETACEANS

The heart of mammals is an organ, subdivided into four chambers, and comparable to a rhythmically double pump, with separate routes for pulmonary and systemic circulation. The right half of the heart pumps the blood that returns from the body to the lungs; here carbon dioxide diffuses out and oxygen diffuses in the blood through the alveolar and capillary membranes. Blood enters the left half of the heart, where it is pumped, through the aorta, throughout the body (Cozzi et al. 2017; Fernández Rodríguez, Caballero Cansino, and Jaber Mohamad 2005).

The heart of cetaceans has the general appearance of that of terrestrial mammals, with some noteworthy differences. For example, the heart of dolphins is larger and flatter than that of most terrestrial mammals (Figure 2), due to the shape of the thorax and the pressure it withstands; the thorax of dolphins is wide and short, and the ribs are connected to the small sternum by bony appendages, being this shape of thorax, in fact, more similar to the human

anatomy (except for the virtual absence of rib cartilages in dolphins), than to other mammals. Therefore, it has an oval almost circular outline, with wider (but shorter) ventricles, a slightly rotated axis, and being the auricles flat with their ventral edges surpassing the coronary sulcus separating atria from ventricles (Cozzi et al. 2017).





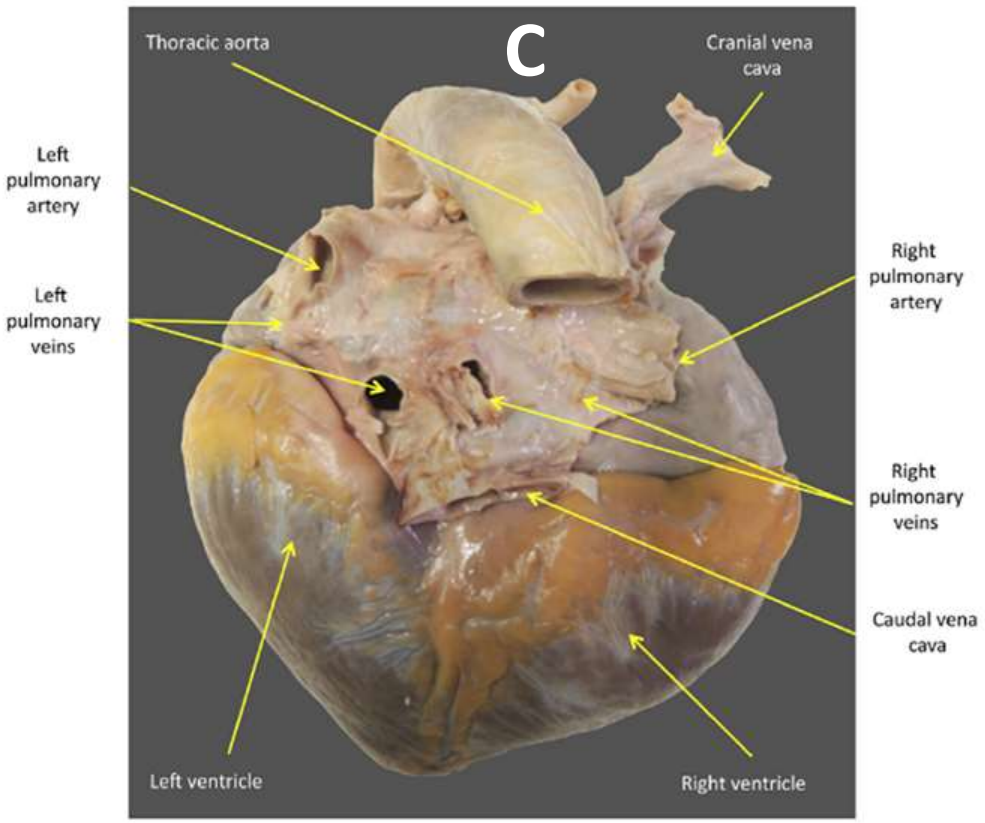


Figure 2. Views of the heart of a dolphin. (A) Ventral view of the heart of a young common bottlenose dolphin (*Tursiops truncatus)*. (B) Ventral view of the heart of an adult common bottlenose dolphin (*Tursiops truncatus)*. (C) Dorsal view of the heart of an adult common bottlenose dolphin (*Tursiops truncatus)*. Figures obtained from the book Anatomy of Dolphins.

Histologically, the cardiac muscle in cetaceans is no different from that of terrestrial mammals. Cardiac muscle fibers (cardiomyocytes) are short, less thick than skeletal striated and uninucleated, with the nucleus in the center of the cells. Three typical regions are distinguished: epicardium, myocardium and endocardium. The outermost layer, which covers externally the myocardium, is the epicardium (visceral serous pericardium). The myocardium is the middle contractile muscular layer of the heart, and is by far the thickest layer of the organ. Here, besides the cardiomyocytes (Figure 3A), it is also possible to observe the Purkinje fibers (Figure 3B), likewise known as cardiac conduction fibers (modified heart cells that transmit nerve impulses). And, finally, the inner layer of the heart is referred to as endocardium, which lines the ventricles and atria completely and covers the cardiac valves and associated structures (Eurell and Frappier 2013; Fernández Rodríguez et al. 2005).

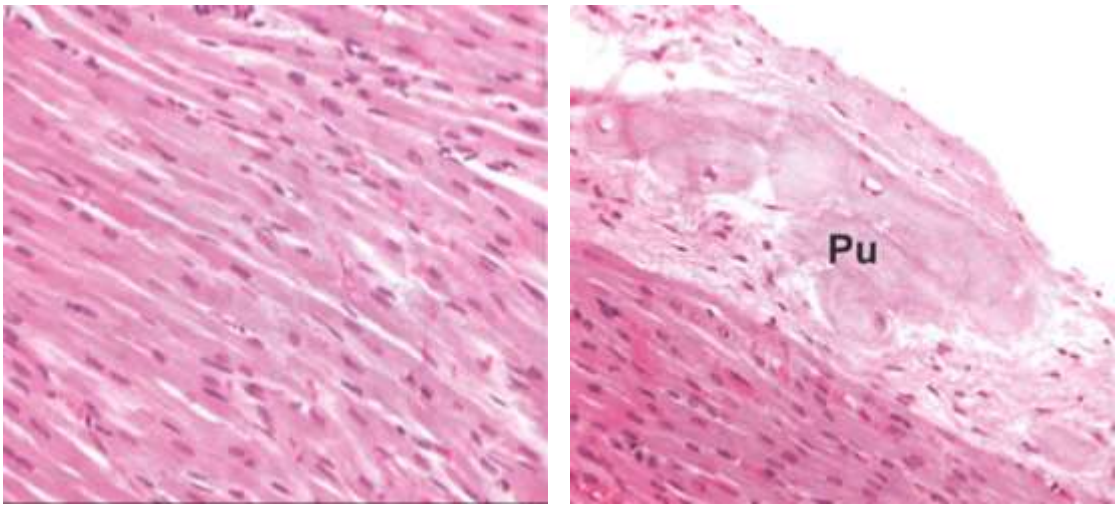


Figure 3. Histology of cardiac muscle. (A) Detail of the cardiac muscle fibers (cardiomyocytes) that constitute the myocardium. (B) Detail of the subendocardial Purkinje fibers (Pu). Figures obtained from the book Atlas de Histología de Peces y Cetáceos.

4.4 GEOGRAPHIC STUDY AREA

According to Law 41/2010, of December 29, the Spanish marine environment is divided in the following marine regions and sub-regions:

a) Northeast Atlantic Region with the Sub-region of the Bay of Biscay and the Iberian coasts, and also with the Macaronesian Atlantic sub-region of the Canary Islands.
b) Mediterranean Sea Region.

To facilitate the application of this law, on the previous marine regions and sub-regions the following subdivisions were established, called marine demarcations, which constitute the spatial scope on which it will be developed each marine strategy:

a) Noratlantic marine demarcation: marine environment in which Spain exercises sovereignty or jurisdiction between the limit of jurisdictional waters between Spain and France in the Bay of Biscay and the northern boundary of jurisdictional waters between Spain and Portugal.
b) South Atlantic marine demarcation: marine environment in which Spain exercises sovereignty or jurisdiction between the limit of jurisdictional waters between Spain and Portugal in the Gulf of Cádiz and the meridian that passes through the Cape of Espartel.
c) Marine demarcation of the Strait and Alborán: marine environment in which Spain it exercises sovereignty or jurisdiction between the meridian that passes through the end of Espartel and an imaginary line with 128 ° orientation with respect to the meridian that passes through the Cape of Gata, and marine environment in which Spain exercises sovereignty or jurisdiction in the area of Ceuta, Melilla, the Chafarinas Islands, the Perejil islet, Peñones de Vélez de la Gomera and Alhucemas and the island of Alborán.
d) Levantino-Balearic marine demarcation: marine environment in which Spain exercises sovereignty or jurisdiction between an imaginary line with 128 ° orientation with respect to the meridian that passes through the end of Gata, and the limit of jurisdictional waters between Spain and France in the Gulf of León.
e) Canary marine demarcation: marine environment in which Spain exercises sovereignty or jurisdiction around the Canary Islands.

4.4.1 CANARY ISLANDS

The Canary Islands are a Spanish archipelago that belongs to the outermost regions of the European Union. The 7 main islands are located in the North Atlantic Ocean near Europe and North Africa, within the biogeographical region of the Macaronesia, which also includes the islands of Azores, Madeira, Savage Islands and Cape Verde and are, from east to west, the island of Lanzarote, Fuerteventura, Gran Canaria, Tenerife, La Gomera, La Palma and El Hierro (Carrillo 2007).

Due to the biodiversity of this archipelago, the Canary Islands were designated as a Particularly Sensitive Sea Area for the International Maritime Organization in 2005 and four of the Canary Islands were declared a Unesco World Biosphere Reserve with several protected areas. In addition, 12 Special Areas of Conservation are currently designated, under the European Habitats Directive, for the conservation of the bottlenose dolphin (*Tursiops truncatus*) and the loggerhead sea turtle (*Caretta caretta*) in the Canary Islands (Tobeña et al. 2014).

a) Features of the archipelago

The geographical position of the Canary Islands in the Atlantic East Node, its volcanic origin, the influence of sea currents and trade winds provide favourable conditions for marine life. The islands act as a screen against the Canary trade winds and the cold current of the Canaries, which runs through the archipelago from north to south, creating food-producing habitats that in turn support numerous species of oceanic habits (Fundación Mapfre Guanarteme 2011).

The availability of food in both deep and surface waters is the determining factor that makes the Canary Islands the region of the planet with the greatest diversity of cetaceans. (Fundación Mapfre Guanarteme 2011).

b) Cetaceans species observed in the archipelago

Nutrient richness is primarily responsible for the great biodiversity of cetaceans that can be observed in the Canary Islands, having registered, from sightings and/or strandings, 30 species (7 mysticetes and 23 odontocetes). Thus, the Canarian waters not only provide

permanent residence for some populations of these species, but they also serve as a migratory route to countless others.

From the infraorder Mysticeti we have:

a) Family Balaenidae:
   - *Eubalaena glacialis*, North Atlantic right whale (only known from sightings)

b) Family Balaenopteridae:
   - *Balaenoptera musculus*, Blue whale (only known from sightings)
   - *Balaenoptera physalus*, Fin whale
   - *Balaenoptera borealis*, Sei whale
   - *Balaenoptera edeni*, Bryde's whale
   - *Balaenoptera acutorostrata*, Common minke whale
   - *Megaptera novaengliae*, Humpback whale

From the infraorder Odonteci there is:

a) Family Ziphiidae
   - *Ziphius cavirostris*, Cuvier's or goose-beaked whale
   - *Mesoplodon densirostris*, Blainville's or dense-beaked whale
   - *Mesoplodon europaeus*, Gervais' beaked whale
   - *Mesoplodon bidens*, Sowerby's beaked whale (only known from strandings)
   - *Mesoplodon mirus*, True's beaked whale (only known from strandings)
   - *Hyperoodon ampullatus*, North Atlantic bottlenose whale (only known from strandings)

b) Family Kogiidae
   - *Kogia breviceps*, Pygmy sperm whale
   - *Kogia sima*, Dwarf sperm whale (only known from strandings)

c) Family Physeteridae
   - *Physeter macrocephalus*, Sperm whale

d) Family Delphinidae
   - *Delphinus delphis*, Short-beaked common dolphin
   - *Tursiops truncatus*, Common bottlenose dolphin
   - *Stenella coeruleoalba*, Striped dolphin
   - *Stenella frontalis*, Atlantic spotted dolphin
   - *Stenella longirostris*, Spinner dolphin (only known from strandings)

- *Lagenodelphis hosei*, Fraser's dolphin (only known from strandings)
- *Grampus griseus*, Risso's dolphin or gray grampus
- *Steno bredanensis*, Rough-toothed dolphin
- *Globicephala macrorhynchus*, Short-finned pilot whale
- *Globicephala melas*, Long-finned pilot whale (only known from strandings)
- *Orcinus orca*, Killer whale or orca
- *Pseudorca crassidens*, False killer whale
- *Feresa attenuate*, Pygmy killer whale (only known from sightings)

e) Family Phocoenidae

- *Phocoena phocoena*, Harbor or common porpoise (only known from strandings)

4.4.2 ANDALUSIA

Andalusia is a bridge between two continents, Africa and Europe, and a meeting point between the Atlantic and the Mediterranean.

Located south of the Iberian Peninsula being the southernmost point of the European Continent. The northern limit is marked by Sierra Morena that breaks the Castilian plateau to the north and the Guadalquivir depression to the south. The western limit is determined by the Guadiana river that separates the province of Huelva from Portugal.

To the south, the presence of the Atlantic Ocean, which bathes the coasts of Huelva and Cádiz, and of the Mediterranean Sea, which does the same with the coasts of Cádiz, Málaga, Granada and Almería mark the southern limit. The eastern limit is determined by the contact between Almería with the Mediterranean Sea and the Spanish Levante.

The autonomous community of Andalusia includes 3 (one of them only partially) of the 5 marine demarcations described in Law 41/2010 mentioned above: South Atlantic marine demarcation, marine demarcation of the Strait and Alborán and Levantino-Balearic marine demarcation (in this case, a small portion of this last demarcation would be within the geographical limits of Andalusia, from Cabo de Gata to the limit with the region of Murcia).

a) Features of the area

The diversity, extension and ecological wealth of the Andalusian territories brings together the highest peaks of the Iberian Peninsula in the Sierra Nevada, extensive wetlands, thick and shady forests, volcanic deserts and stretches of coastline with hardly any human trace.

The marine environment and the Andalusian coast stand out for their high biological and landscape diversity and their ecological complexity. Its geographical location favours a great wealth of habitats and species, a fact that gives its waters and seabed the greatest biodiversity values of the European seas. The almost 1100 kilometres of coastline of the Autonomous Community, one of the largest in the entire Spanish territory, offer multiple positive externalities, which, apart from their exceptional environmental values, include economic, social and cultural aspects of various kinds, sometimes with divergent interests and intervention formulas with the conservation of the natural environment.

The region of the Strait of Gibraltar is considered an ecosystem of great biological richness, due to the confluence of the surface Atlantic and deeper Mediterranean waters, which together with the different hydrographic and topographic characteristics, make this marine region extremely important at the level of its ecosystems. It is considered the dynamic engine of the biodiversity of the western Mediterranean and probably the one with the greatest biological diversity of the European coasts (Raga and Pantoja 2004).

On the other hand, the Alborán Sea has oceanographic characteristics that make it an area of transition between the Atlantic Ocean and the Mediterranean Sea.

b) Cetaceans species observed in the area

The 3 marine demarcations included in Andalusia are characterized by a high number (19 in total) of cetacean species present, more specifically 4 mysticetes and 15 odontocetes.

From the infraorder Mysticeti we have:

a) Family Balaenopteridae:
   - *Balaenoptera physalus*, Fin whale
   - *Balaenoptera edeni*, Bryde's whale (only known from strandings)
   - *Balaenoptera acutorostrata*, Common minke whale

- *Megaptera novaengliae*, Humpback whale

From the infraorder Odonteci there is:

a) Family Ziphiidae
   - *Ziphius cavirostris*, Cuvier's or goose-beaked whale
   - *Mesoplodon densirostris*, Blainville's or dense-beaked whale (only known from strandings)
   - *Mesoplodon europaeus*, Gervais' beaked whale (only known from strandings)
   - *Hyperoodon ampullatus*, North Atlantic bottlenose whale (only known from sightings)
b) Family Kogiidae
   - *Kogia breviceps*, Pygmy sperm whale
   - *Kogia sima*, Dwarf sperm whale (only known from strandings)
c) Family Physeteridae
   - *Physeter macrocephalus*, Sperm whale
d) Family Delphinidae
   - *Delphinus delphis*, Short-beaked common dolphin
   - *Tursiops truncatus*, Common bottlenose dolphin
   - *Stenella coeruleoalba*, Striped dolphin
   - *Grampus griseus*, Risso's dolphin or gray grampus
   - *Globicephala melas*, Long-finned pilot whale
   - *Orcinus orca*, Killer whale or orca (only known from sightings)
   - *Pseudorca crassidens*, False killer whale (only known from sightings)
e) Family Phocoenidae
   - *Phocoena phocoena*, Harbor or common porpoise

MATERIALS & METHODS

5.1 DETERMINATION OF THE STUDY ANIMALS

The average number of strandings in the Canary Islands to which this research unit has access is around 45 cetaceans per year. In Andalusia there is, more or less, 150 cetaceans strandings per year, but a great number of them we do not have access to. Nonetheless, this research unit only has access to a phew number of samples, which are sent by stranding network responsible in Andalusia.

Based on Arbelo et al. (2013), Díaz-Delgado et al. (2018) and Maldonado (2015), integrating the growing scientific bibliography and experience gained in the last 20 years of pathological studies of cetaceans stranded in the Canary Islands and in Andalusia, as well as the integration of knowledge of comparative pathology of human and domestic animals, of free and exotic life, we have considered that the pathological entities that will best allow us to characterize catecholamine cardiomyopathies in stranded cetaceans will be the following:

5.1.1 ACTIVE/LIVE STRANDING

This pathological entity is quite important since, previous studies show that in the Canary Islands from 1999 to 2005, 27% (37 animals out of a total of 138) of the animals stranded alive and 13% (30 animals out of a total of 236) in 2006 to 2012, and that in Andalusia from 2011 to 2014, 27% (28 animals out of a total of 104) of the animals were live-stranded (Arbelo et al. 2013; Díaz-Delgado et al. 2018; Maldonado 2015). Thus, it is extremely important to study the biochemical parameters related to SCMP in cetaceans, since it is in our interest to be able to make a return to the marine environment of these animals with the minimum possible damage. Likewise, the measurement of these parameters at the time of stranding and the use of drugs, which control the negative effects of the release of high concentrations in the bloodstream of biomarkers of heart damage, are possibly one of the key points in the release and return rate of these animals.

5.1.2 SHIP COLLISIONS

As previously mentioned, the collision of vessels with cetaceans is a documented and significant global threat, with a rapid increase in its incidence, due to the development of

maritime traffic on an international scale, the increase in speed (more than 35 knots) and the size of the fleet. Prior studies show that in the Canary Islands from 1999 to 2005, 6% (8 animals out of a total of 138) of the animals died due to ship collision and 10% (24 animals out of a total of 236) in 2006 to 2012, and that in Andalusia from 2011 to 2014, 2% (2 animals out of a total of 104) of the animals died because of a collision with a vessel (Arbelo et al. 2013; Díaz-Delgado et al. 2018; Maldonado 2015). Therefore, it is extremely important to study this pathological entity to advance the knowledge and the possible increase in technology in large ships which could help to reduce collisions, since ship strikes are more common than what was thought of, being in some cases, an important conservation problem, on certain threatened cetaceans' species.

5.1.3 BYCATCH

As formerly described, fishing interactions, more specifically accidental catches (bycatch), are one of the biggest threats to many populations, not just cetaceans. In 1994 it was calculated that of the total catches, made by the Spanish drift fleet operating in the Strait of Gibraltar, only 7% corresponded to the target species (swordfish), the remaining 93% accounted for being accidental catches (cetaceans, turtles and elasmobranchs) (Maldonado 2015). Preceding studies show that in the Canary Islands from 1999 to 2005 and during the period 2006 to 2012, 7 and 10 animals died due to bycatch, respectively. In Andalusia from 2011 to 2014, 4 animals died through bycatch (Arbelo et al. 2013; Díaz-Delgado et al. 2018; Maldonado 2015). Although much progress has been made in the diagnosis of this type of interaction, which implies capture and subsequent drowning of the animal in the nets, it remains a challenge to show that the cause of death was the interaction with fishing gear. For this reason, it is extremely important to study this pathological entity, in order to provide another piece of knowledge to facilitate the diagnosis of this cause of death.

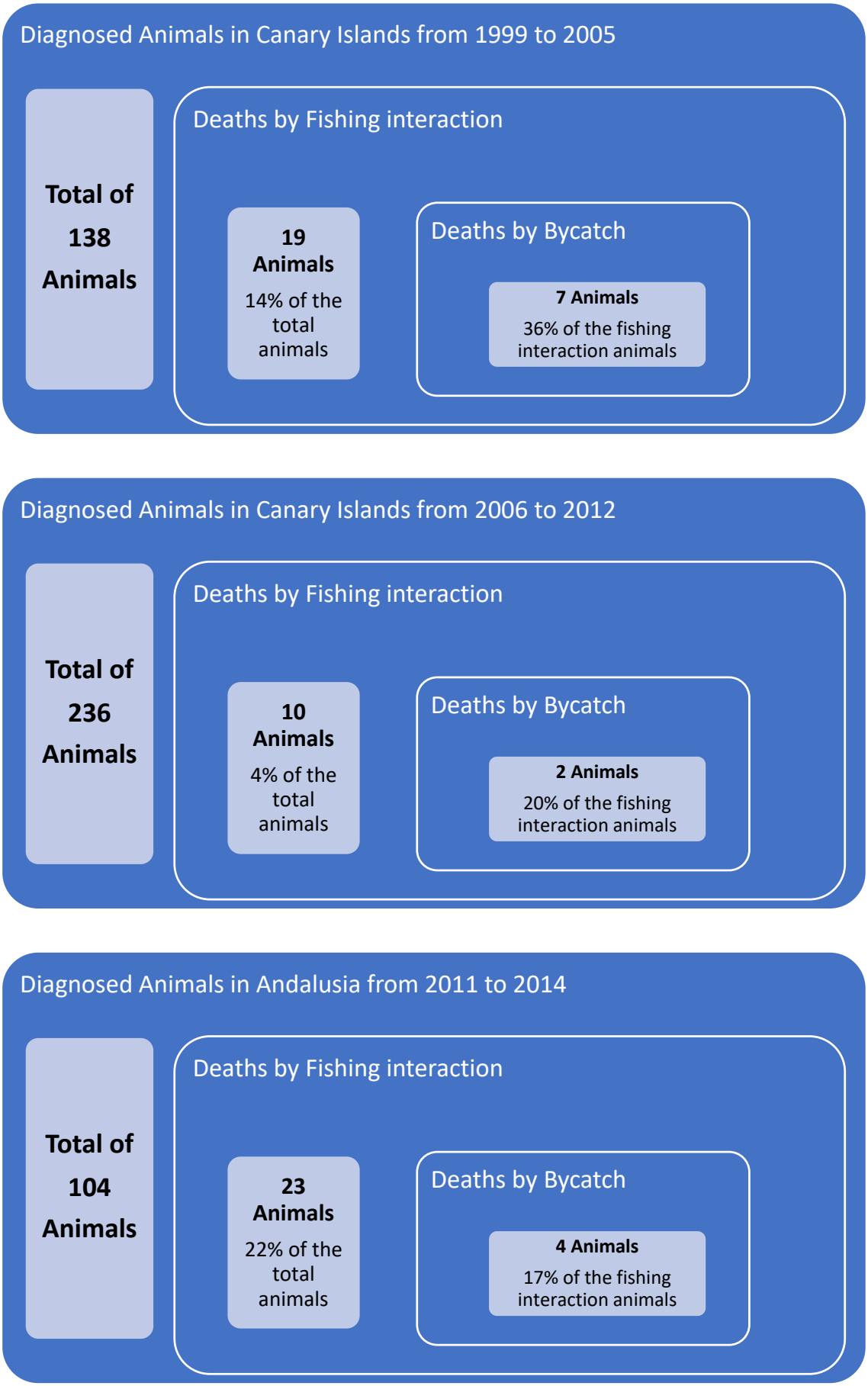

Diagnosed Animals in Canary Islands from 1999 to 2005
Total of 138 Animals
Deaths by Fishing interaction
19 Animals
14% of the total animals
Deaths by Bycatch
7 Animals
36% of the fishing interaction animals
Diagnosed Animals in Canary Islands from 2006 to 2012
Total of 236 Animals
Deaths by Fishing interaction
10 Animals
4% of the total animals
Deaths by Bycatch
2 Animals
20% of the fishing interaction animals
Diagnosed Animals in Andalusia from 2011 to 2014
Total of 104 Animals
Deaths by Fishing interaction
23 Animals
22% of the total animals
Deaths by Bycatch
4 Animals
17% of the fishing interaction animals

Figure 4. Diagram representing the number of animals which died due to bycatch (in comparison with the animals which died by fishing interaction and with the total of animals diagnosed) in Canary Islands from 1999 to 2005; 2006 to 2012 and in Andalusia from 2011 to 2014.

5.1.4 ANIMALS INCLUDED IN THE STUDY

To perform this study, we used heart samples from stranded cetaceans (whales and dolphins) in different geographical locations of the Canary archipelago and in Andalusia. These tissues are stored in the tissue bank of the Cetacean Research Unit, Division of Animal Histology and Pathology, University Institute of Animal Health and Food Safety, belonging to the University of Las Palmas de Gran Canaria (ULPGC).

Each specific objective of this work had a particular material selection explained as follow:

a) Characterization of Stress Cardiomyopathy

The first phase of this specific objective consisted of the selection of all the animals (n=148) that stranded alive and subsequently died previous to or during the rescue and rehabilitation (n=83), that died due to ship collision (n=32), and died from entanglement in fishery lines (n=33). All of these animals were stranded on the coast of the Canary Islands from 2000 to 2016 and on the coast of Andalucía from 2011 to 2014.

At the end of the second phase, a total of 67 animals (48 stranded alive, 7 died from ship collision and 12 from bycatch) were selected for their “very fresh”, “fresh” and “moderate autolysis” conservation state without prior freezing. Previous studies have shown that animals with advanced autolysis or previously frozen present a great number of false negatives when using histochemical and immunohistochemical techniques.

The following scheme illustrates our sample selection and the total number of animals evaluated in each phase (Figure 4).

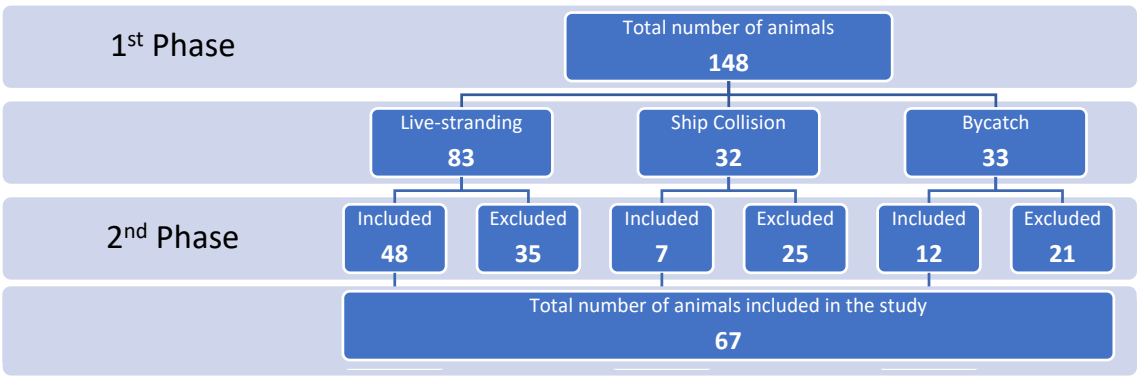


Figure 5. Diagram representing the selection of animals in each phase of the first specific objective of the present work.

b) Correlation of the blood values detected for the biochemical markers of acute cardiac muscle damage with the pathological findings identified histologically

The first phase of this specific objective consisted in determining a normal baseline range for cardiac troponin I in cetaceans, specifically bottlenose dolphins, with a 95% probability through measurements obtained from captive animals (n=5) at a local zoo (Palmitos Park). To achieve this, a total of 20 blood samples were collected, in June, September and December of 2018 and March of 2019 (4 samples of each individual, 1 sample in each month).

In the second phase, a total of 9 animals that stranded alive were included in this study. All of these animals were stranded on the coast of the Canary Islands from beginning of 2016 to June of 2019. Blood samples were collected from these animals, being one pre-mortem and the rest post-mortem since the animals subsequently died previous to or during the handling, restraint, transport, and/or rescue/rehabilitation (n=8).

During the third phase, a total of 7 animals were selected, from the animals included (n=9) in the previous phase, for the histological and histochemical study, since we were not able to perform a necropsy in 2 animals, as one was released back to sea and the other was used for anatomical research purposes.

At the fourth phase of the study, we decided to eliminate 2 animals (from the prior phase) as they had been frozen and as mentioned before, animals previously frozen present a great number of false negatives when using immunohistochemical techniques. Therefore, at the end of this phase, a total of 5 animals were studied with the use of the immunohistochemical technique for the detection of different markers.

In the following scheme we present the different stages presented formerly (Figure 5).

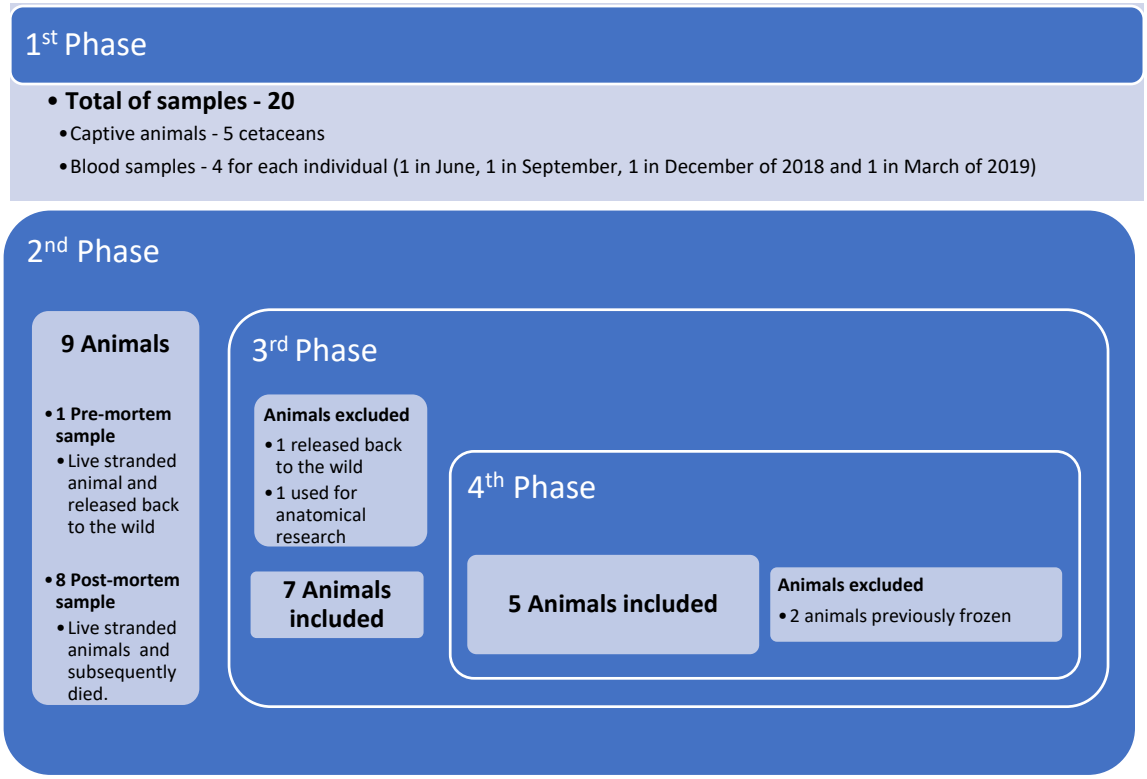


Figure 6. Diagram representing the different phases of the second specific objective of the present study.

c) Application of the knowledge obtained previously in the diagnosis of specific clinical cases

To achieve this specific objective, 2 animals were carefully chosen based on specific characteristics.

The first animal was selected based on the fact that it was a live-stranded specimen belonging to the *Mysticeti* infraorder, and unfortunately, the description of pathological entities and/or causes of death in the infraorder Mysticeti are still scarce.

The second report is centred on a live-stranded animal which was in rehabilitation during a certain period of time, allowing to obtain consecutive blood samples in vivo.

Both case studies were carried out following specific stages, represented in Figure 7.

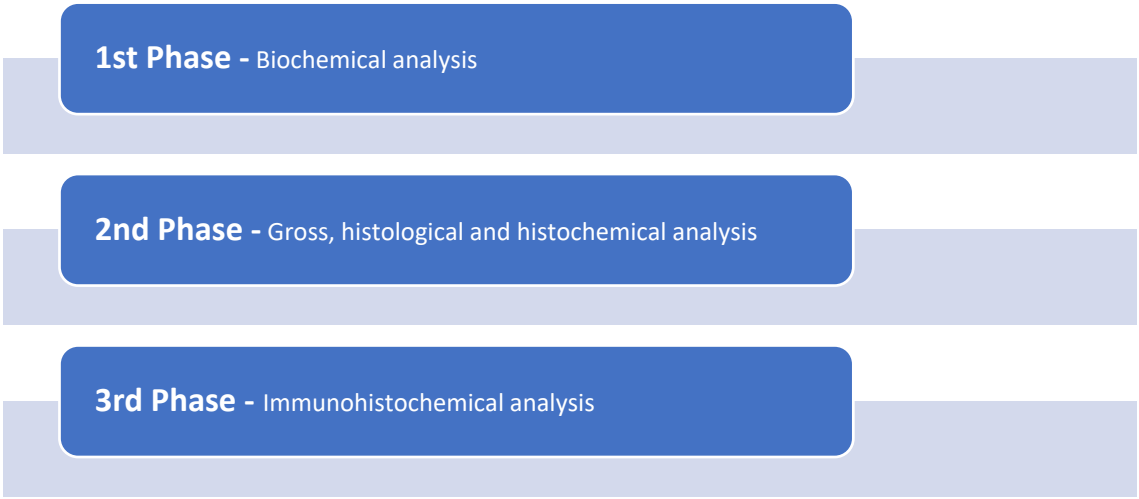


Figure 7. Diagram representing the different phases carried out in each case study of the third specific objective of the present work.

5.2 TECHNIQUES USED IN THE STUDY

5.1.1 BLOOD COLLECTION

Needles 18-21 gauge (2.5 to 9 cm) will be required, depending on the size of the animal. In addition to the central veins of the tail and the central arteriovenous complex in the dorsal fin, locations of the pectoral fins and caudal peduncle have been used, but there is an increased risk of thrombovasculitis if these sites are sampled repeatedly (Barnett, Knight, and Steven n.d.).

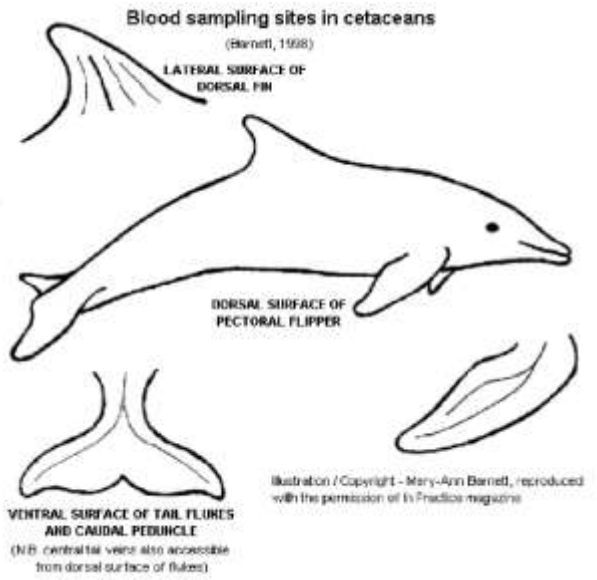


Figure 8. The diagram below illustrates the commonly used sampling sites. Figure obtained from the BDMLR - Marine Mammal Medic Handbook.

The blood sample is collected to a gel tube (without anticoagulant), it is allowed to clot and then is centrifuge at about 3500-4000 rotations for 5 minutes, two times, to obtain the serum. At least 0.5 to 1 ml of serum should be taken for measurements.

The serum can be stored for a maximum of one week in refrigeration (4º C) or up to one month in freezing (-20º C) until it is sent and processed in a laboratory.

5.1.2 NECROPSY TECHNIQUE

Necropsies are performed in order to better understand the cause of death. In marine animals these may indicate the cause of the stranding or other mortalities. Thus, they allow a

series of crude observations, which provide differential diagnoses (Díaz-Delgado 2015; Godinho 2010; Hernández 2007; De La Fuente Marquez 2011).

Subsequent investigations, such as histopathology, also allow several potential diagnostics to be ruled out until a definitive etiology is established (Díaz-Delgado 2015; Godinho 2010; Hernández 2007; De La Fuente Marquez 2011).

Regardless of whether it is a common chronic disease, interaction with fishing nets, or an emerging zoonosis, the constant performance of necropsies allows monitoring viral outbreaks/trends in the marine population (Díaz-Delgado 2015; Godinho 2010; Hernández 2007; De La Fuente Marquez 2011).

Due to logistical issues, the necropsy can be performed in a variety of locations, however it is noteworthy that the chosen site will obviously condition the level of compliance with the protocol stipulated for performing an appropriate diagnostic necropsy (Díaz-Delgado 2015; Godinho 2010; Hernández 2007; De La Fuente Marquez 2011). Nonetheless, it is important to remark that in the stranded animals, whose conservation conditions and logistical circumstances allowed, a complete or partial necropsy was performed.

The basic protocol for performance of a cetacean necropsy was outlined and published by the European Cetacean Society by Thijs Kuiken and Manuel García Hartmann (Proceedings of the first ECS workshop on Cetacean Pathology: Dissection Techniques and Tissue Sampling. Leiden, Netherlands, 13-14 September 1991. Newsletter # 17 special issue). This protocol has been updated and innovated as it addresses procedures used for necropsy of domestic terrestrial mammals from Professor King and colleagues' Necropsy of Domestic Animals book, along with some modifications of the same procedures referenced in the manual published by Geraci and Lounsbury in 2005 (Necropsy Book, Veterinary School, Cornell University, New York, US 1989).

Therefore, depending on the experience gained and as new pathologies not previously described are found, the relevant modifications or priorities are introduced in the protocols.

Tissue samples of approximately 2-3 cm^3 are collected during the necropsy. All of these samples are taken from both apparently healthy organs and those that may be injured and stored in 4% buffered formaldehyde for further histopathology (Díaz-Delgado 2015; Godinho 2010; Hernández 2007; De La Fuente Marquez 2011).

In order to make an accurate diagnosis of the cause and / or death of the cetacean under study, it is equally important to collect duplicates of specific organs, which are frozen at -80° C

for chemical contaminants, virological and/or bacteriological studies, as well as mucosal isotopes and fluid samples in the body cavities, urine, blood, vitreous humor or others needed (Díaz-Delgado 2015; Godinho 2010; Hernández 2007; De La Fuente Marquez 2011).

5.1.3 SAMPLE PREPARATION

Histology is the study of body tissues and cells, namely their constituents. Cells cannot be seen with the naked eye, so the primary tool used to study them is the microscope. It produces enlarged images of cells and also increases the contrast for detail resolution (Ovalle and Nahirney 2008).

Most cells absorb very little light, so staining is required to increase light absorption. Cells and tissues first undergo sequential stages of histological processing. Fixation (by aldehydes) and dehydration (by alcohols) are followed by inclusion in paraffin or plastic resin. Specimen cuts are obtained on a microtome, followed by dye staining (Ovalle and Nahirney 2008).

a) Fixation and dehydration

The samples, after being fixed for at least 24 h in 10% buffered formaldehyde, are cut (approximately 1.5cm x 0.3 cm) and placed in cassettes for automatic processing and later inclusion in paraffin.

In short, this processing consists of the following:

Reactive	Time	Temperature
Formalin	3 h	Ambience
Alcohol 70%	1 h	Ambience
Alcohol 96% I	1 h	Ambience
Alcohol 96% II	1 h	Ambience
Alcohol 100% I	1 h	Ambience
Alcohol 100% II	1 h and 30 minutes	Ambience
Alcohol 100% III	1 h and 30 minutes	Ambience
Xylene I	1 h	Ambience
Xylene II	1 h	Ambience

Reactive	Time	Temperature
Xylene III	1 h and 30 minutes	Ambience
Paraffin I	1 h and 30 minutes	59ºC
Paraffin II	1 h and 30 minutes	59ºC

b) Paraffin Inclusion

Once the aforementioned process is completed, the paraffin blocks with melting point of 56° C were made and then cut with a microtome. The cuts are extended into distilled water at 45ºC to be transferred to crystal slides. Haematoxylin and Eosin staining (HE) was performed using untreated slides, while the slides to be subjected to the Periodic Acid Shiff (PAS) stain, Phosphotungstic Acid-Haematoxylin (PTAH) stain and Masson's trichrome stain technique were previously treated with Poly-L-lysine. for a better fixation of the cuts on the crystal blades.

5.1.4 HISTOLOGICAL AND HISTOCHEMICAL TECHNIQUES

Histological and histochemical techniques provide different but complementary views of cells and thus a useful morphological basis that may assist in understanding cellular function in health and disease (Ovalle and Nahirney 2008).

For all the stains used in this study, the cuts were subjected to heat in the oven at a temperature of 100ºC for 30 minutes and subsequently deparaffinised and hydrated by the following process:

Reactive	Time	Temperature
Xylene I	2 Minutes	Ambience
Xylene II	2 Minutes	Ambience
Alcohol 100% I	2 Minutes	Ambience
Alcohol 100% II	2 Minutes	Ambience
Alcohol 70%	2 Minutes	Ambience
Distilled water I	2 Minutes	Ambience
Distilled water II	2 Minutes	Ambience
Distilled water III	2 Minutes	Ambience

a) Haematoxylin and Eosin (HE) Staining

Paraffin sections, 5 µm thick, are routinely stained with HE and examined under an optical microscope (MO). Cell nuclei (which are rich in nucleic acids such as DNA and RNA) have an affinity for haematoxylin (a basic dye) staining a bluish kind, and later characterized as basophils. In contrast, cell cytoplasm and extracellular matrix typically have an affinity for eosin (an anionic dye), stain pink, and are described as eosinophils (or acidophilus) (Ovalle and Nahirney 2008).

After dewaxing and hydration, mentioned a priori, the following automatic procedure is performed:

Reactive	Time	Temperature
Harris Haematoxylin	15 minutes followed by a brief wash in distilled water for 1 minute	Ambience
Hydrochloric Alcohol	4 brief passes followed by a quick 1 minute water wash	Ambience
Ammoniacal Water	15 passes	Ambience
Running Water	15 minutes	Ambience
Eosin	4 minutes oozing well at the end and absorbing excess dye with paper	Ambience

Once the HE staining process is complete, the samples are dehydrated according to the subsequent automated process:

Reactive	Time	Temperature
Alcohol 96% I	2 Minutes	Ambience
Alcohol 96% II	2 Minutes	Ambience
Alcohol 100% I	2 Minutes	Ambience
Alcohol 100% II	2 Minutes	Ambience
Xylene I	2 Minutes	Ambience
Xylene II	2 Minutes	Ambience
Xylene III	2 Minutes	Ambience

After the previous procedure, the coverslips are applied using DPX, which favours permanent mounting and observation under the optical microscope.

b) Periodic Acid Schiff (PAS) Staining

In opposition to organelles - cytoplasmic structures that perform all cellular functions - inclusions are relatively inert, dispensable and transient components, which vary in type and distribution. Usually metabolic by-products or stored nutrients include glycogen, lipid droplets and pigment granules (Ovalle and Nahirney 2008).

Glycogen is a D-glucose polymer, stored mainly in the cytoplasm of hepatocytes and skeletal muscle cells, and in smaller amounts in cells of other tissues. Glycogen synthesis, storage and degradation occur rapidly as needed. Glycogen is not normally visualized in routine sections from which it is removed unless preserved and stained by special techniques such as PAS staining (Ovalle and Nahirney 2008).

The 4 µm thick sections are submitted to heat and they are deparaffinated and hydrated as mentioned above in the HE stain, followed by the PAS technique.

Reactive	Time	Temperature
Periodic Acid 0.5%	5 Minutes	Ambience
Distilled Water	1 wash	Ambience
Schiff Reactive	10 Minutes	Ambience
Running Water	5 Minutes	Ambience
Harris Haematoxylin	6 Minutes	Ambience
Running Water	10 Minutes	Ambience

Once the above procedure is completed, the samples are dehydrated and mounted as explained in HE staining.

c) Phosphotungstic Acid-Haematoxylin (PTAH) Staining

PTAH is a mix of haematoxylin with phosphotungstic acid, used in histology for staining. It stains some tissue in contrasting colours in a way similar to HE stain, as phosphotungstic acid binds to tissue proteins. It is used to show gliosis in the central nervous system, tumours of skeletal muscles, and fibrin deposits in lesions. Muscle is stained blue-black to dark brown,

connective tissue is pale orange-pink to brownish red, fibrin and neuroglia stain deep blue, coarse elastic fibers show as purple, and bone and cartilage obtain yellowish to brownish red colour (Carson and Cappellano 2009).

This technique is ideal for demonstrating striated muscle fibers and mitochondria, often without a counterstain. As such, it is used to identify contraction bands, as seen in contraction band necrosis (Carson and Cappellano 2009; Vargas, Sampson, and Schoen 1999).

After dewaxing and hydration, mentioned a priori, of the 5 µm thick tissue sections, the following procedure is performed:

Reactive	Time	Temperature
Zenker fixer	24 h	37ºC
Running Water	Abundantly followed by 2 brief passes in distilled water	Ambience
Iodinated solution of Langeron	10 minutes	Ambience
Sodium Thiosulfate	5 minutes followed by 5 minutes in distilled water	Ambience
PTAH	2 h	60ºC

Once the PTAH staining process is complete, the samples are dehydrated and mounted according to the process explained in HE staining.

d) Masson's trichrome Staining

Collagen fibers are the elements most frequently found in connective tissue. They have a basic support function and are synthesized by multiple cellular elements of the organism. From the point of view of conventional histology, all techniques for staining collagen fibers that are classically grouped under the denominations of trichrome colorations, have type I collagen, which forms the thick collagen fibers existing in extracellular spaces and organic stroma. Most recipes produce red keratin and muscle fibers, blue or green collagen and bone, light red or pink cytoplasm, and dark brown to black cell nuclei (Garcia del Moral 1993).

The 4 µm thick sections are deparaffinated and hydrated as mentioned above in the Haematoxylin and Eosin (H&E) stain, followed by the Masson's trichrome technique.

Reactive	Time	Temperature
Harris Haematoxylin	8 minutes followed by brief pass in distilled water	Ambience
Picric Acid	15 minutes followed by brief pass in distilled water	Ambience
Acid Fuchsin Biebrich Scarlet	6 minutes followed by brief pass in distilled water	Ambience
Phosphomolibedic-Phosphotungstic Acid	2 passes of 3 minutes each followed by brief pass in distilled water	Ambience
Aniline Blue	6 minutes followed by brief pass in distilled water	Ambience
1% acetic acid	2 passes of 5 minutes each followed by brief pass in distilled water	Ambience

Once the above procedure is completed, the samples are dehydrated and mounted as explained in HE staining.

5.1.5 IMMUNOHISTOCHEMICAL TECHNIQUE

Immunohistochemistry is the most common application of immunostaining. It involves the process of selectively identifying antigens (proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues (Ramos-Vara and Miller 2014).

Immunohistochemical staining is widely used in the diagnosis of abnormal cells such as those found in cancerous tumors. Specific molecular markers are characteristic of particular cellular events such as proliferation or cell death (apoptosis). Immunohistochemistry is also widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue (Whiteside and Munglani 1998).

Tissue samples are typically sliced at a range of 3 µm. The slices are then mounted on slides treated with VECTABOND® reagent for a better tissue section adhesion. Tissue sections rest approximately 48h to dry well. After this, the samples require certain steps to make the epitopes available for antibody binding, including deparaffinization and antigen retrieval, as followed:

Reactive	Time	Temperature
Xylene I	10 minutes	Ambience
Xylene II	5 minutes	Ambience
Xylene III	5 minutes	Ambience
Alcohol 100%	5 minutes	Ambience
Block of Endogenous Peroxidase [1]	30 minutes while stirring in a container where light does not pass	Ambience
Alcohol 100%	5 minutes	Ambience
Alcohol 96%	5 minutes	Ambience
Alcohol 70%	5 minutes	Ambience
Distilled Water I	5 minutes while stirring	Ambience
Distilled Water II	5 minutes while stirring	Ambience
PBS Solution [2] I	5 minutes while stirring	Ambience
PBS Solution [2] II	5 minutes while stirring	Ambience

(1) Hydrogen Peroxide 30% solution from Honeywell Fluka™ with reference 008-003-00-9 mixed with Methanol (EMSURE®) from Merck with reference 1.06009.1000 (6 ml of Hydrogen Peroxide in 194 ml of Methanol). It is important to add the Hydrogen Peroxide right before using the solution. (2) For 2 litters of distilled water we add: 2.96 g of Sodium Hydrogen Phosphate anhydrous from PanReac AppliChem ITW Reagents with reference 131679.1211 with 0.86 g of Potassium di-Hydrogen Phosphate from PanReac AppliChem ITW Reagents with reference 121509.1210 and finally 14.4 g of Sodium Chloride from PanReac AppliChem ITW Reagents with reference 131679.1211.

For formalin-fixed paraffin-embedded tissues, antigen-retrieval is often necessary, and involves pre-treating the sections with heat or protease. These steps may make the difference between the target antigens staining or not staining. Depending on the tissue type and the method of antigen detection, endogenous biotin or enzymes may need to be blocked or quenched, respectively, prior to antibody staining. Although antibodies show preferential avidity for specific epitopes, they may partially or weakly bind to sites on nonspecific proteins (also called reactive sites) that are similar to the cognate binding sites on the target antigen. A great amount of non-specific binding causes high background staining which will mask the detection of the target antigen. To reduce background staining samples are incubated with a

buffer that blocks the reactive sites to which the primary or secondary antibodies may otherwise bind. Common blocking buffers include normal serum, non-fat dry milk, BSA, or gelatin. Methods to eliminate background staining include dilution of the primary or secondary antibodies, changing the time or temperature of incubation, and using a different detection system or different primary antibody. Quality control should include a minimum tissue known to express the antigen as a positive control, as well as, two negative controls being one sample of tissue known not to express the antigen, and the test tissue probed in the same way with omission of the primary antibody (or better, absorption of the primary antibody) (Ramos-Vara and Miller 2014).

The previously mentioned methods and the subsequent protocol are performed as followed:

Reactive	Time	Temperature
Pre-treatment	Variable	Ambience
PBS Solution I	5 minutes while stirring	Ambience
PBS Solution II	5 minutes while stirring	Ambience
Normal Serum Incubation	30 minutes (starting to count after putting the serum in the last crystal)	In the humidity chamber at ambience temperature
Primary Antibody Incubation	At least 18 h	In the humidity chamber inside refrigerator
Humidity Chamber	1 h	Ambience
PBS Solution I	5 minutes while stirring	Ambience
PBS Solution II	5 minutes while stirring	Ambience
PBS Solution III	5 minutes while stirring	Ambience
Secondary Antibody Incubation	30 minutes (starting to count after putting the serum in the first crystal)	In the humidity chamber at ambience temperature
PBS Solution I	5 minutes while stirring	Ambience
PBS Solution II	5 minutes while stirring	Ambience
Third Antibody Incubation [1]	30 minutes (starting to count after putting the serum in the last crystal)	In the humidity chamber at ambience temperature in a room without light
PBS Solution I	5 minutes while stirring	Ambience

Reactive	Time	Temperature
PBS Solution II	5 minutes while stirring	Ambience
Acetate Solution [2]	10 minutes while stirring	Ambience
Reveal Solution [3]	Variable [4]	Ambience
Running Water	10 minutes	Ambience
Mayer's Haematoxylin	10 minutes	Ambience
Running Water	10 minutes	Ambience
Distilled Water	5 minutes	Ambience
Mounting [5]	-	Ambience

(1) Tissue samples are visualized using the VECTASTAIN Elite ABC-Peroxidase Kit from Vector Laboratories with reference PK-6100. In 5000 µl of PBS solution we put 2 drops (100 µl) of reagent A and 2 drops (100 µl) of reagent B. (2) For 1 litter of Acetate you need: 1.2 ml of Acetic Acid Glacial from PanReac AppliChem ITW Reagents with reference 131008.1211 mixed with 210 ml of distilled water and 10.75 g Sodium Acetate Anhydrous from PanReac AppliChem ITW Reagents with reference 131633.1210 mixed with 790 ml of distilled water. (3) 0.5 g of 3-Amino-9-Ethylcarbazole from Sigma with reference A5754 mixed with 10 ml of N-N-Dimethylformamide from Sigma with reference D4551. The room should have minimal light when preparing this solution and due to the toxicity of these reagents, it should be prepared under the gas extraction fan and the personal must use a protective gas mask. Mix this solution in a container where light does not pass (container that is not translucent). Add 140ml of Acetate solution. Filter the solution. Add 150 µl of Hydrogen Peroxide right before using the solution. (4) 3 minutes in the case of Myoglobin and Cardiac Troponin C and 5 minutes for Fibrinogen and Cardiac Troponin I. (5) Mount the crystals with the tissue samples with glass cover and Faramount Aqueous Mounting Medium from Dako with reference S3025.

All of the information about the pre-treatment for the different antibodies used in this study and all the primary and secondary antibodies are summarized in the following table.

ANTIGEN RETRIEVAL	SERUM	SOURCE	DILUTION	PRIMARY ANTIBODY	SOURCE	HOST	TYPE	DILUTION	SECONDARY ANTIBODY	SOURCE	DILUTION
Citrate buffer (1)	Swine serum (3)	Dako (4)	10% (5)	Myoglobin (6)	Abcam (10)	Rabbit	Polyclonal	1 in 200 (11)	Polyclonal Swine Anti-Rabbit Immunoglobulins (15)	Dako (4)	1 in 200 (16)
Citrate buffer (1)	Swine serum (3)	Dako (4)	10% (5)	Fibrinogen (7)	Abcam (10)	Rabbit	Polyclonal	1 in 50 (12)	Polyclonal Swine Anti-Rabbit Immunoglobulins (15)	Dako (4)	1 in 200 (16)
Citrate buffer (2)	Swine serum (3)	Dako (4)	10% (5)	Troponin I (8)	Abcam (10)	Rabbit	Polyclonal	1 in 25 (13)	Polyclonal Swine Anti-Rabbit Immunoglobulins (15)	Dako (4)	1 in 200 (16)
Citrate buffer (2)	Swine serum (3)	Dako (4)	10% (5)	Troponin C (9)	Abcam (10)	Rabbit	Monoclonal	1 in 250 (14)	Polyclonal Swine Anti-Rabbit Immunoglobulins (15)	Dako (4)	1 in 100 (17)

(1) Citrate buffer, pH 6.0, 7 minutes at 96ºC. (2) Citrate buffer, pH 6.0, 20 minutes at 100ºC. (3) Dako Swine serum (Normal) (X090110-8). (4) Dako (Glostrup, Denmark). (5) Dilution of 10 µl of serum in 90 µl of PBS. (6) Anti-Myoglobin antibody (ab187506). (7) Anti-Fibrinogen antibody (ab34269). (8) Anti-Cardiac Troponin I antibody (ab47003). (9) Anti-Cardiac Troponin C antibody (ab137130). (10) Abcam (Cambridge, United Kingdom). (11) Dilution of 1 µl of primary antibody in 199 µl of serum at 1% in PBS (dilution of 1 µl of serum in 99 µl of PBS). (12) Dilution of 1 µl of primary antibody in 49 µl of serum at 1% in PBS (dilution of 1 µl of serum in 99 µl of PBS). (13) Dilution of 1 µl of primary antibody in 24 µl of serum at 1% in PBS (dilution of 1 µl of serum in 99 µl of PBS). (14) Dilution of 1 µl of primary antibody in 249 µl of serum at 1% in PBS (dilution of 1 µl of serum in 99 µl of PBS). (15) Dako Polyclonal Swine Anti-Rabbit Immunoglobulins/Biotinylated (E035301-2). (16) Dilution of 1 µl of secondary antibody in 199 µl of serum at 1% in PBS (dilution of 1 µl of serum in 99 µl of PBS). (17) Dilution of 1 µl of secondary antibody in 99 µl of serum at 1% in PBS (dilution of 1 µl of serum in 99 µl of PBS).

RESULTS
&
DISCUSSION

PUBLICATIONS

6.1 CHARACTERIZATION OF STRESS CARDIOMYOPATHY

The first specific objective of this work, which consisted in characterizing the SCMP in cetaceans, such as injuries resulting from extreme stress responses, was achieved using histological, histochemical and immunohistochemical approaches on a total of 67 animals which live-stranded or died due to ship collisions and interactions with fisheries (bycatch).

This resulted in the publication of an article (as followed) titled ***Stress cardiomyopathy in stranded cetaceans: a histological, histochemical and immunohistochemical study***, on September 2019 in the *Veterinary Record,* Volume 185, Issue 22 (doi: 10.1136/vr.105562), having this journal an impact factor of 2.101 in 2018 and therefore being a Q1 in the veterinary category.

Stress cardiomyopathy in stranded cetaceans: a histological, histochemical and immunohistochemical study

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ABSTRACT

Background Free-living cetaceans are exposed to a wide variety of stressful situations, including live stranding and interaction with human beings (capture myopathy), vessel strikes, and fishing activities (bycatch), which affect their wellbeing and potentially lead to stress cardiomyopathy (SCMP).

Methods Here, the authors aimed to characterise SCMP of stranded cetaceans as an injury resulting from extreme stress responses, based on pathological analyses (histological, histochemical and immunohistochemical). Specifically, the authors examined heart samples from 67 cetaceans found ashore (48 live strandings, seven dead from ship collision and 12 dead from bycatch) on the coast of Spain, more specifically in the Canary Islands from 2000 to 2016 and Andalusia from 2011 to 2014.

Results The microscopic findings were characterised by vascular changes, acute or subacute cardiac degenerative necrotic lesions, interstitial myoglobin globules, and infiltration of inflammatory cells. Immunohistochemically, cardiac troponin I, cardiac troponin C and myoglobin were depleted, along with fibrinogen being expressed in the degenerated/necrotic cardiomyocytes. A perivascular pattern was also identified and described in the damaged cardiomyocytes.

Conclusions This study advances current knowledge about the pathologies of cetaceans and their implications on conserving this group of animals by reducing mortality and enhancing their treatment and subsequent rehabilitation to the marine environment.

Introduction

Today, stress is a very commonly used word and is present in the day-to-day life; however, all life forms have evolved mechanisms to cope with stressful situations. Yet it is viewed as a pervasive modern-day killer.[1] Stress is defined as an internal (physiological or psychogenic) or environmental stimulus that initiates adaptive changes or stress responses to an animal.[2] However, it is commonly defined as the biological response caused when a person or animal perceives a threat to their homeostasis, with the threat being the stressor.[3]

The adaptive responses to a stressor might vary depending on the species and/or individual, but also because of a complex interplay of genetic factors and previous experiences. However, stress responses always lead to a number of changes, including alterations to behaviour, the endocrine system and the autonomic system. These changes induce significant physiological effects on the body by activating the hypothalamus-pituitary-adrenal axis, with the consequent production and release of catecholamines (adrenaline and noradrenaline), glucocorticoid and mineralocorticoid.[4–6] Endogenous catecholamines are neurotransmitters which in physiological plasma concentrations exert a positive inotropic action on the heart. However, when these are present in large doses, they induce myocardial injury, both to animals and human beings.[7–9] Under conditions of stress,

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large amounts of catecholamines are released from the sympathetic nerve endings and adrenal medulla, with exposure to these high concentrations producing coronary spasms, arrhythmias, contractile dysfunction, myocardial cell damage and extensive necrosis.[10]

There is increasing awareness of a unique cardiac syndrome in human pathology, which has been described as the 'Apical Ballooning Syndrome', 'Takotsubo Disease', and ampulla or stress cardiomyopathy (SCMP), otherwise known as the 'Broken Heart Syndrome' in the popular press.[11] This syndrome is a reversible cardiomyopathy (CMP) that typically occurs after an emotional or physical stress event. Its clinical presentation cannot be distinguished from a myocardial infarction.[11 12] Several cases have been reported globally.[13–22] SCMP is an important differential diagnosis of an acute myocardial infarction; consequently, it is under-recognised and often misdiagnosed.[11]

The pathophysiology of this syndrome appears to be related to an excess of plasma catecholamines, which are triggered by stressful conditions. Histological findings are consistent with catecholamine cardiotoxicity, due to ischaemia-reperfusion injury, both in human myocardial samples and in experimental models of SCMP.[11 12 23–32] The crucial role of myocardial ischaemia in Takotsubo syndrome has been identified, with most cases occurring in patients with a risk factor for endothelial dysfunction. This dysfunction is characterised by an imbalance between vasoconstricting and vasodilating factors, which might explain the propensity to epicardial and/or microvascular coronary artery spasm of the disease.[33 34]

Marine mammals, in this case cetaceans, present the same basic response to stress as human beings and other mammals. Once the central nervous system perceives a threat, a biological or defence response is developed.[35] This response is well documented in marine mammals.[36–40] Although physiological stress has some benefits, an extreme or prolonged response, with release of catecholamines and sustained high blood cortisol and aldosterone levels, could potentially damage skeletal and heart muscles.[35] Consequently, cardiac lesions seem to play a central role in marine mammals, with cetaceans being particularly prone to develop SCMP, probably due to the characteristics of their cardiovascular adaptations, conforming to the requirements of diving metabolism.[35 39 41–43]

Free-living cetaceans are at daily risk to a wide variety of stressful situations, of both natural and anthropogenic origin, that influence their wellbeing. In recent decades, efforts have been made to reduce the impact of some human activities on cetaceans. Unfortunately, whales and dolphins continue to be threatened, for example, by active (live) stranding, ship collision and bycatch. These different pathological entities have acute stress as the central aetiopathogenesis, potentially leading to the death of the animal or seriously aggravating a previous disease situation. These issues impact the subsequent rehabilitation of animals, reducing the effectiveness of therapy and limiting successful recovery.[39 40 42 44–49]

Acute and stressful deaths of cetaceans, such as occurs in live strandings, might be attributed to the so-called 'stress response syndrome' or 'alarm reaction'. The involved mechanisms are thought to be analogous to capture myopathy in terrestrial mammals and are typically referred in the literature, particularly in prolonged rescues and rehabilitation efforts.[39 42 45 50] The response consists of local to generalised vasospasm and vasodilation (catecholamines, neurogenic shock, impeded venous flow return by body compression), concurring with the SCMP in human beings, direct traumatic injury of the muscle and viscera, and reperfusion damage.[39 42 45]

The collision of ships with cetaceans is recognised as an important global threat, with a rapid increase in its incidence in recent years. For individuals that suffer severe trauma after impact with a boat, death generally occurs in an acute form, due to haemorrhagic shock (hypovolaemic), mirroring the SCMP process in human beings, and directly impacting vital organs (eg, section of the spinal cord). Alternatively, chronic trauma occurs, which can lead to the development of sepsis or generalised weakness.[44 45 47]

Fishing gears also present a major threat to cetaceans, worldwide, because of their widespread use.[51] When trapped in fishing nets, small whales, dolphins and porpoises often die because they are not strong enough to break free or surface to breathe.[52] Captured cetaceans typically exhibit haemodynamic changes to shock, as it happens in the SCMP in human beings, due to submersion or drowning (asphyxia). Often, macroscopic or histopathological lesions are evident in the cardiovascular and respiratory systems.[44–46 48]

Here, the authors aimed to characterise SCMP of stranded cetaceans as an injury resulting from extreme stress responses. Cetaceans from live strandings, ship collisions and bycatch were analysed via histological, histochemical and immunohistochemical approaches.

Materials and methods

One hundred and forty-eight cetaceans of 20 different species, including small and large odontocetes and mysticetes, were initially included in this study. Of these, 83 were stranded alive (and subsequently died previous to or during the rescue and rehabilitation), 32 died due to ship collision and 33 died from entanglement in fishery lines. All of these animals were stranded on the coast of Spain, more specifically in the Canary Islands from 2000 to 2016 and Andalusia from 2011 to 2014. The pathological findings in the different organs and causes of death (stranded alive, ship collision and entanglement in fishery lines) of the 148 animals are described in annual reports.[44 45 53]

A complete postmortem examination was performed following the standard protocol published by the European Cetacean Society,[54] with some additional procedures detailed in the manual *Marine Mammals Ashore: A Field Guide for Strandings*.[50] The conservation state of the bodies was also determined following the parameters and classifications established by these two protocols. Thus, out of the initial 148 animals, the authors identified 67 animals (48 stranded alive, seven dead from ship collision and 12 from bycatch) belonging to 19 species that had a very fresh, fresh and moderate autolysis conservation state. Previous studies have shown that animals with advanced autolysis or that were previously frozen present a great number of false negatives when using histochemical and immunohistochemical techniques.

The samples from the 67 animals were fixed and processed following standard histological procedures. Specifically, 4 µm of samples was used for haematoxylin and eosin (HE) and periodic acid-Schiff staining, and 5 µm was used for phosphotungstic acid haematoxylin (PTAH) and Masson's trichrome techniques. The basic epidemiological data about the animals are presented in online supplementary file 1. Tissue sections of 3 µm were immunolabelled using antimyoglobin, antifibrinogen, anti-cardiac troponin I (cTnI) and anti-cardiac troponin C (cTnC) primary antibodies, and were visualised using the VECTASTAIN Elite ABC-Peroxidase Kit (PK-6100) from Vector Laboratories (Peterborough, UK). The immunohistochemical methodology is summarised in table 1. The negative control for the immunohistochemical techniques was performed without the primary antibody on a serial heart section. The positive control for myoglobin and fibrinogen was a cetacean heart sample of a striped dolphin (previously published to have been stranded alive and to have developed capture myopathy due to capture and human interaction during the rehabilitation process).[39 42] For cTnI and cTnC, the authors used a heart sample from a pig, with no apparent acute macroscopic and/or histological lesions.

The sections submitted to the different techniques were examined in a blind manner by three veterinary pathologists (NC, ES and PH). The sections were evaluated for vascular changes (congestion, haemorrhages and interstitial oedema); acute degenerative changes (contraction band necrosis, wavy fibres, perinuclear vacuolisation, cytoplasmic hypereosinophilia and pyknotic nuclei); and presence of interstitial myoglobin globules and infiltration of inflammatory cells. The extent of cardiac lesions was judged subjectively as absent, mild, mild to moderate, moderate, moderate to severe, and severe.

The amount of damage present to cells was confirmed as antemortem when it was accompanied with immunohistochemically demonstrated depletion of myoglobin together with intrafibrillar fibrinogen deposition.

Table 1 Summary of the immunohistochemical methodology used in this study

Antigen retrieval	Serum	Source	Dilution	Primary antibody	Source	Host	Type	Dilution	Secondary antibody	Source	Dilution
Citrate buffer (1)	Swine serum (3)	Dako (4)	10% (5)	Myoglobin (6)	Abcam (10)	Rabbit	Polyclonal	1 in 200 (11)	Polyclonal Swine Anti-Rabbit Immunoglobulins (15)	Dako (4)	1 in 200 (16)
Citrate buffer (1)	Swine serum (3)	Dako (4)	10% (5)	Fibrinogen (7)	Abcam (10)	Rabbit	Polyclonal	1 in 50 (12)	Polyclonal Swine Anti-Rabbit Immunoglobulins (15)	Dako (4)	1 in 200 (16)
Citrate buffer (2)	Swine serum (3)	Dako (4)	10% (5)	Troponin I (8)	Abcam (10)	Rabbit	Polyclonal	1 in 25 (13)	Polyclonal Swine Anti-Rabbit Immunoglobulins (15)	Dako (4)	1 in 200 (16)
Citrate buffer (2)	Swine serum (3)	Dako (4)	10% (5)	Troponin C (9)	Abcam (10)	Rabbit	Monoclonal	1 in 250 (14)	Polyclonal Swine Anti-Rabbit Immunoglobulins (15)	Dako (4)	1 in 100 (17)

(1) Citrate buffer, pH 6.0, seven minutes at 96ºC. (2) Citrate buffer, pH 6.0, 20 minutes at 100ºC. (3) Dako Swine serum (normal) (X0901l0-8). (4) Dako (Glostrup, Denmark). (5) Dilution of 10 µl of serum in 90 µl of PBS. The serum is incubated in a humidity chamber for half an hour. (6) Antimyoglobin antibody (ab187506). (7) Antifibrinogen antibody (ab34269). (8) Anti-cardiac troponin I antibody (ab47003). (9) Anti-cardiac troponin C antibody (ab137130). (10) Abcam (Cambridge, UK). (11) Dilution of 1 µl of primary antibody in 199 µl of serum at 1% in PBS (dilution of 1 µl of serum in 99 µl of PBS). The primary antibody is incubated in a humidity chamber for at least 18 hours, inside the refrigerator. (12) Dilution of 1 µl of primary antibody in 49 µl of serum at 1% in PBS (dilution of 1 µl of serum in 99 µl of PBS). The primary antibody is incubated in a humidity chamber for at least 18 hours, inside the refrigerator. (13) Dilution of 1 µl of primary antibody in 24 µl of serum at 1% in PBS (dilution of 1 µl of serum in 99 µl of PBS). The primary antibody is incubated in a humidity chamber for at least 18 hours, inside the refrigerator. (14) Dilution of 1 µl of primary antibody in 249 µl of serum at 1% in PBS (dilution of 1 µl of serum in 99 µl of PBS). The primary antibody is incubated in a humidity chamber for at least 18 hours, inside the refrigerator. (15) Dako Polyclonal Swine Anti-Rabbit Immunoglobulins/Biotinylated (E035301-2). (16) Dilution of 1 µl of secondary antibody in 199 µl of serum at 1% in PBS (dilution of 1 µl of serum in 99 µl of PBS). The secondary antibody is incubated in a humidity chamber for half an hour. (17) Dilution of 1 µl of secondary antibody in 99 µl of serum at 1% in PBS (dilution of 1 µl of serum in 99 µl of PBS). The secondary antibody is incubated in a humidity chamber for half an hour.

Table 2 Classification for the absence or presence, in different degrees, of vascular changes (congestion, interstitial oedema and haemorrhage) in the 67 selected animals, from the 148 initially examined animals, belonging to the distinct pathological entities and causes of death, in this case ship collision (seven animals), bycatch (12 animals) and live-stranded (48 animals)

	Total number of cases		Ship collision cases		Bycatch cases		Live stranding cases	
	Animals (n)	Percentage	Animals (n)	Percentage	Animals (n)	Percentage	Animals (n)	Percentage
Congestion								
Absence	22	32.8	5	71.4	2	16.7	15	31.3
Mild	23	34.3	2	28.6	6	50.0	15	31.3
Mild to moderate	6	9.0	0	0	2	16.7	4	8.3
Moderate	13	19.4	0	0	1	8.3	12	25.0
Moderate to severe	1	1.5	0	0	1	8.3	0	0
Severe	2	3.0	0	0	0	0	2	4.2
Interstitial oedema								
Absence	41	61.2	3	42.9	6	50.0	32	66.7
Mild	12	17.9	3	42.9	4	33.3	5	10.4
Mild to moderate	5	7.5	0	0	2	16.7	3	6.3
Moderate	5	7.5	0	0	0	0	5	10.4
Moderate to severe	3	4.5	1	14.3	0	0	2	4.2
Severe	1	1.5	0	0	0	0	1	2.1
Haemorrhage								
Absence	53	79.1	6	85.7	11	91.7	36	75.0
Mild	8	11.9	1	14.3	1	8.3	7	14.6
Mild to moderate	1	1.5	0	0	0	0	1	2.1
Moderate	1	1.5	0	0	0	0	1	2.1
Moderate to severe	1	1.5	0	0	0	0	0	0
Severe	3	4.5	0	0	0	0	3	6.3

Results

Histologically, the major cardiac lesions associated with stress were (1) vascular changes (table 2), which presented as congestion, haemorrhage and interstitial oedema; (2) acute degenerative changes, which were attributed to cardiotoxicity by catecholamines (table 3) and consisted of contraction band necrosis, wavy fibres, cytoplasmic hypereosinophilia and perinuclear vacuolisation; and (3) the presence of interstitial myoglobin globules (table 4) and infiltration of inflammatory cells (table 5).

Vascular congestion was identified by swelling and the light occlusion of capillaries with blood (figure 1). This phenomenon was observed in 45 out of 67 animals (67.2 per cent). This condition was mild to moderate in the individuals that died due to bycatch (10 out of 12 animals, 83.3 per cent) and live-stranded (33 out of 48 animals, 68.8 per cent). Individuals that died from ship collision (two out of seven animals, 28.6 per cent) displayed a mild degree of congestion.

Out of the 67 animals, 26 (38.9 per cent) had fibres separated by spaces, representing interstitial oedema. Overall, it was mild to moderate for all pathological categories. It was particularly noticeable in four of the seven animals (57.2 per cent) that died from ship collisions, six of the 12 animals (50.0 per cent) that died due to bycatch, and 16 of the 48 animals (33.4 per cent) that were stranded alive. Eight out of the 67 animals (11.9 per cent) with interstitial oedema also had haemorrhages.

Only haemorrhages could be visualised grossly and microscopically (figure 1). They were classified as endocardial, myocardial and epicardial, and were primarily observed in the endocardium. One out of the 67 animals (1.5 per cent) exclusively exhibited gross haemorrhages, four (6.0 per cent) exhibited gross and microscopic haemorrhages, and 10 (14.9 per cent) only presented microscopic haemorrhages that were of a mild to moderate degree. Microscopically, one of the seven animals (14.3 per cent) that died from ship collisions exhibited a moderate to severe degree of haemorrhage, 12 out of the 48 animals (25.0 per cent) that were stranded alive exhibited a mild to moderate degree, and one out of 12 animals (8.3 per cent) that died due to bycatch exhibited a mild degree.

Contraction band necrosis is the focal hypercontraction and lysis of contractile filaments in small groups of myocardial cells. The resulting dense hypereosinophilic bands run transversely through the myocyte (figure 2). This acute degenerative change was observed, mainly with a mild to moderate degree, in 33 out of the 67 animals (49.3 per cent). Some cases had lesions that were more marked at the subendocardial and subepicardial level. Of note, all of the animals that died from ship collisions (seven individuals, 100 per cent) presented contraction band necrosis that was moderate to severe, followed by the animals that were stranded alive (25 out of 48 animals, 52.1 per cent) which displayed mild to moderate necrosis, and animals that died due to bycatch (one out of 12 animals, 8.3 per cent) with a mild degree of necrosis.

Twenty-nine out of the 67 animals (43.3 per cent) primarily exhibited a mild degree of long and thin undulated fibres, termed wavy fibres (figure 3). Wavy

Table 3 Classification for the absence or presence, in different degrees, of acute degenerative changes (contraction band necrosis, wavy fibres, hypereosinophilia and cytoplasmic vacuolisation) in the 67 selected animals, from the 148 initially examined animals, belonging to the distinct pathological entities and causes of death, in this case ship collision (seven animals), bycatch (12 animals) and live-stranded (48 animals)

	Total number of cases		Ship collision cases		Bycatch cases		Live stranding cases	
	Animals (n)	Percentage	Animals (n)	Percentage	Animals (n)	Percentage	Animals (n)	Percentage
Contraction band necrosis								
Absence	34	50.7	0	0	11	91.7	23	47.9
Mild	17	25.4	3	42.9	1	8.3	13	27.1
Mild to moderate	4	6.0	0	0	0	0	4	8.3
Moderate	2	3.0	0	0	0	0	2	4.2
Moderate to severe	2	3.0	1	14.3	0	0	1	2.1
Severe	8	11.9	3	42.9	0	0	5	10.4
Wavy fibres								
Absence	38	56.7	2	28.6	6	50.0	30	62.5
Mild	18	26.9	3	42.9	5	41.7	10	20.8
Mild to moderate	3	4.5	0	0	0	0	3	6.3
Moderate	7	10.4	2	28.6	1	8.3	4	8.3
Moderate to severe	1	1.5	0	0	0	0	1	2.1
Severe	0	0	0	0	0	0	0	0
Hypereosinophilia								
Absence	0	0	0	0	0	0	0	0
Mild	10	14.9	1	14.3	6	50.0	3	6.3
Mild to moderate	9	13.4	2	28.6	1	8.3	6	12.5
Moderate	26	38.8	2	28.6	5	41.7	19	39.6
Moderate to severe	12	17.9	0	0	0	0	12	25.0
Severe	10	14.9	2	28.6	0	0	8	16.7
Vacuolar degeneration								
Absence	2	3.0	0	0	1	8.3	1	2.1
Mild	15	22.4	4	57.1	7	58.3	4	8.3
Mild to moderate	7	10.4	1	14.3	1	8.3	5	10.4
Moderate	22	32.8	2	28.6	3	25.0	17	35.4
Moderate to severe	13	19.4	0	0	0	0	13	27.1
Severe	8	11.9	0	0	0	0	8	16.7

fibres were observed, with a mild to moderate degree, in five out of seven animals (71.5 per cent) that died from ship collisions, six out of 12 animals (50.0 per cent) that died from bycatch, and 18 out of 48 animals (37.5 per cent) that were stranded alive.

Hypereosinophilia was easily identified by comparing the region suspected to be necrotic against fibres in the same section. The necrotic fibres presented a more eosinophilic, blue or red (using HE, PTAH and Masson's trichrome technique, respectively) staining than the adjacent normal fibres (figure 4). This acute degenerative change was observed in all animals (67, 100 per cent) from a moderate to severe degree. In some cases, hypereosinophilia was more pronounced at the subendocardial and subepicardial level. All animals that were stranded alive (48 animals in total, 100 per cent) presented an overall moderate to severe degree of hypereosinophilia, along with all animals that died from ship collisions (seven individuals, 100 per cent), which presented a moderate degree, and all animals that died from bycatch (12 animals, 100 per cent), which mostly presented a mild to moderate degree of hypereosinophilia.

Sixty-five out of the 67 animals (96.9 per cent) exhibited some degree of vacuolar degeneration. This acute degenerative change was morphologically characterised by the intracellular accumulation of fluid and lysis of myofibrils. The increase in liquid exerts pressure on the different parts of the cell, resulting in most of the fibres with vacuoles having a pyknotic nucleus (figure 5). In some cases, this lesion was more noticeable at the subendocardial and subepicardial level. Forty-seven out of 48 animals (97.9 per cent) that were stranded alive presented vacuoles in the fibres of a

Table 4 Classification for the absence or presence of interstitial myoglobin globules in the 67 selected animals, from the 148 initially examined animals, belonging to the distinct pathological entities and causes of death, in this case ship collision (seven animals), bycatch (12 animals) and live-stranded (48 animals)

	Total number of cases		Ship collision cases		Bycatch cases		Live stranding cases	
	Animals (n)	Percentage	Animals (n)	Percentage	Animals (n)	Percentage	Animals (n)	Percentage
Interstitial myoglobin globules								
Absence	38	56.7	5	71.4	3	25.0	30	62.5
Presence	29	43.3	2	28.6	9	75.0	18	37.5

Table 5 Classification for the absence or presence, in different degrees, of inflammatory infiltration in the 67 selected animals, from the 148 initially examined animals, belonging to the distinct pathological entities and causes of death, in this case ship collision (seven animals), bycatch (12 animals) and live-stranded (48 animals)

	Total number of cases		Ship collision cases		Bycatch cases		Live stranding cases	
	Animals (n)	Percentage	Animals (n)	Percentage	Animals (n)	Percentage	Animals (n)	Percentage
Inflammatory infiltration								
Absence	50	74.6	5	71.4	8	66.7	37	77.1
Mild	14	20.9	2	28.6	2	16.7	10	20.8
Mild to moderate	1	1.5	0	0	1	8.3	0	0
Moderate	1	1.5	0	0	0	0	1	2.1
Moderate to severe	1	1.5	0	0	1	8.3	0	0
Severe	0	0	0	0	0	0	0	4

moderate to severe degree, all animals (seven animals, 100 per cent) that died from ship collisions had a mild to moderate degree of vacuoles, and 11 of the 12 animals (91.6 per cent) that died from bycatch had a mild degree of vacuoles.

Immunohistochemically, the degenerated/necrotic cardiomyocytes showed homogeneous, intrafibrillar depletion of cTnI, cTnC and myoglobin (figures 6–9). Simultaneously, the authors verified that myoglobin accumulated as globules inside the blood vessels and in the interstitial spaces (figure 10). The myoglobin globules were observed in 29 out of 67 animals (43.3 per cent). Specifically, myoglobin globules were detected in nine out of 12 animals (75.0 per cent) that died from bycatch, 18 out of 48 animals (37.5 per cent) that were stranded alive, and two out of seven animals (28.6 per cent) that died from ship collisions. Damaged cardiomyocytes exhibited various intensities of immunolabelling for fibrinogen. In some cases, cTnI, cTnC and fibrinogen were strongly expressed in the zone next to and inside the contraction band necrosis (figures 7, 9 and 11). However, cTnI, cTnC and myoglobin were so slightly expressed that they disappeared at times (depletion), with fibrinogen expression emerging more intensively. Immunolabelling of uninjured cardiomyocytes produced homogeneous, intracytoplasmic immunolabelling for cTnI, cTnC and myoglobin, and did not stain for fibrinogen.

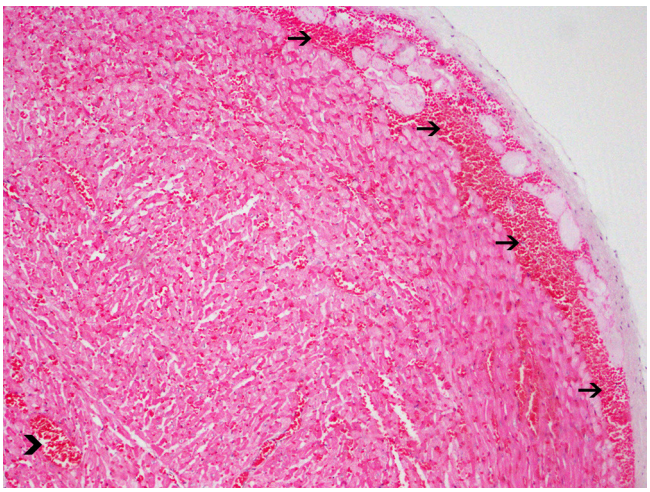

Figure 1 Live-stranded animal: *Kogia breviceps* (reference: i373_07). Endocardial haemorrhage (arrows) and congestion (arrowhead) visualised with haematoxylin and eosin, magnification x 10.

The major cardiac lesions associated with stress (such as the hypereosinophilia and cytoplasmic vacuolisation) were more commonly observed in a multifocal distribution in the rows of cardiomyocytes that were located closest to the blood vessels and that followed the paths. Such cells were damaged or formed groups of damaged cells. Thus, the acute degenerative changes present in the degenerated/necrotic cardiomyocytes had a perivascular pattern (figures 12 and 13). Live-stranded animals presented this pattern more intensively.

In seventeen out of 67 animals (25.37 per cent), different types of cells with a mild degree of inflammation were identified infiltrating zones where haemorrhages, fibrillary rupture of fibres, degeneration and necrosis of cardiac cells were present. Four out of 12 animals (33.3 per cent) that died from bycatch displayed a mild to moderate degree of inflammatory cell infiltration, as well as two out of seven (28.6 per cent) that died from ship collisions and 11 animals out of 48 animals (22.9 per cent) that were stranded alive. Six out of the 67 animals (9.0 per cent) presented a mild degree of infiltration of mononuclear cells. This condition was present to a mild degree in animals that died from ship

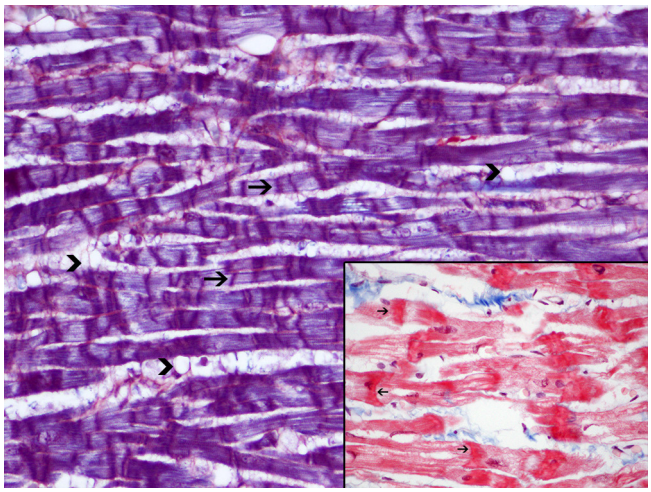

Figure 2 Live-stranded animal: *Kogia breviceps* (reference: i373_07). The contraction band necrosis runs (arrows) transversely through the cardiomyocytes, and it is identified through the higher intensity of colour with different histochemical techniques, in this case with the PTAH technique, magnification x 60. Notice also the presence of myoglobin globules in the interstitial space (arrowheads). Inset: Live-stranded animal: *K. breviceps* (reference: i452_00). Detail of the contraction band necrosis (arrows) with Masson's trichrome, magnification x 60. PTAH, phosphotungstic acid haematoxylin.

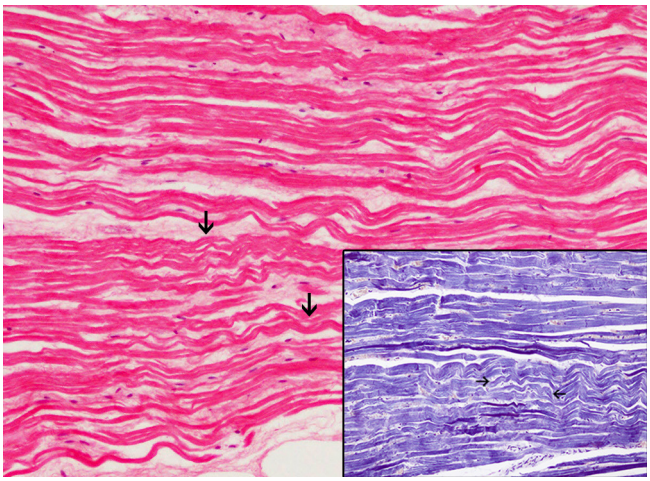

Figure 3 Ship collision animal: *Physeter macrocephalus* (reference: i59_09). Wavy fibres (arrows) which consist of long and thinned undulated fibres. HE technique, magnification x 20. Inset: Live-stranded animal: *Mesoplodon densirostris* (reference: i92_04). Detail of wavy fibres (arrows) with PTAH, magnification x 20. HE, haematoxylin and eosin; PTAH, phosphotungstic acid haematoxylin.

collisions (two out of seven animals, 28.6 per cent) and that were stranded alive (three out of 48 animals, 6.3 per cent). It was present at a moderate to severe degree in animals that died from bycatch (one out of 12 animals, 6.3 per cent). Three out of the 67 animals (4.5 per cent) displayed a mild degree of infiltration by polymorphonuclear cells. This condition was present at mild degree in animals that died from bycatch (two out of 12 animals, 16.7 per cent) and animals that were stranded alive (one out of 48 animals, 2.1 per cent). Twelve out of the 67 animals (17.9 per cent) had a mild degree of infiltration of macrophage cells. This condition was present at a mild to moderate degree in three out of the 12 animals that died from bycatch (24.9 per cent), and at a mild degree in nine of the 48 animals that were stranded alive (18.8 per cent).

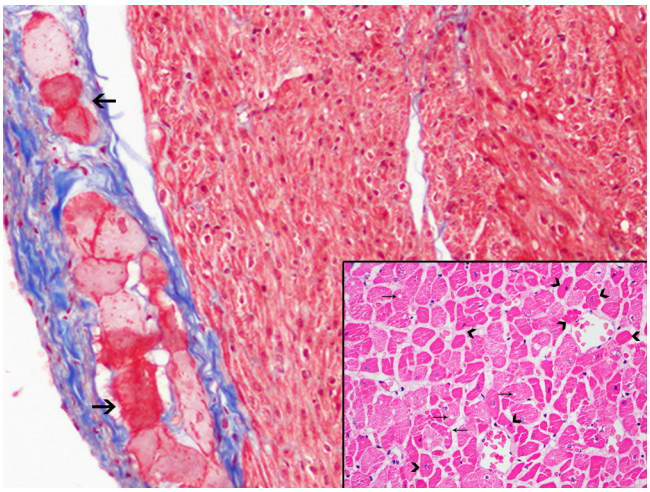

Figure 4 Live-stranded animal: *Pseudorca crassidens* (reference: i102_08). Hypereosinophilia consists of an increased staining of necrotic cardiac cells, such as Purkinje cells (arrows), with different techniques, in this case with Masson's trichrome technique, magnification x 20. Inset: Live-stranded animal: *Mesoplodon densirostris* (reference: i238_03). Hypereosinophilia (arrowheads) of necrotic cardiomyocytes stained with HE, magnification x 40. Furthermore, observe the presence of cytoplasmic vacuolisation in some injured cardiomyocytes (thin arrows). HE, haematoxylin and eosin.

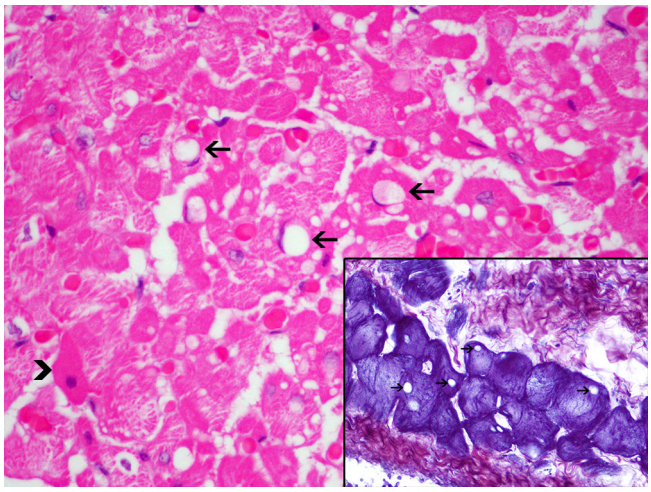

Figure 5 Live-stranded animal: *Kogia breviceps* (reference: i373_07). Detail of the intracytoplasmic vacuoles (arrows) of the cardiomyocytes with HE technique. Moreover, take into consideration the presence of injured cells with hypereosinophilic staining (arrowhead), magnification x 60. Inset: Bycatch animal: *Delphinus delphis* (reference: i263_11). Vacuolar degeneration (arrows) in Purkinje cells, with PTAH technique. Magnification x 40. HE, haematoxylin and eosin; PTAH, phosphotungstic acid haematoxylin.

Discussion

Based on the histological analysis of biopsied and postmortem myocardial tissue of examined specimens (mostly human), SCMP is associated with severe morphological alterations, consisting of vascular changes and acute degenerative lesions.[11 23 24 26–32]

Subendocardial congestion, interstitial oedema and haemorrhages are detected by histological approaches and form part of the pathology of a catecholamine CMP.[29]

Vascular congestion was observed in 45 of the 67 animals (67.2 per cent) in this study, and presented as swelling or capillary light occlusions, resulting from damage to capillary endothelial cells. This condition is normally present in stressful situations.[39 42 45 49 55]

The tissue samples of 26 animals (38.8 per cent) contained fibres separated by spaces, representing interstitial oedema, supporting published literature.[28] Following administration of catecholamines, interstitial oedema is usually associated with subendocardial and

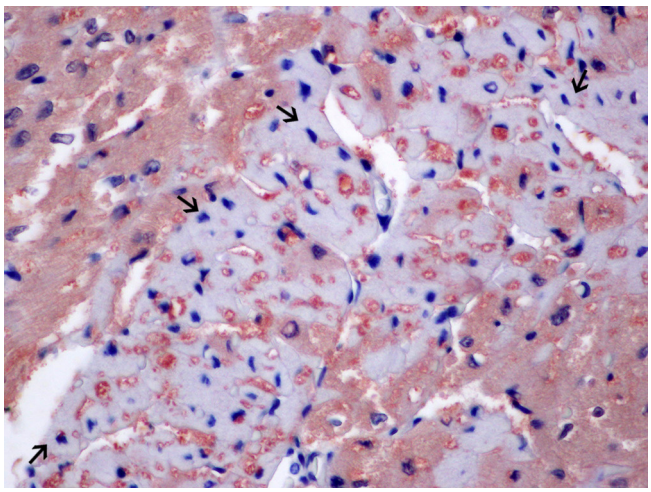

Figure 6 Live-stranded animal: *Delphinus delphis* (reference: i87_03). Degenerated/necrotic cardiomyocytes (arrows) show intrafibrillar depletion of myoglobin. Immunohistochemical technique: antimyoglobin, magnification x 60.

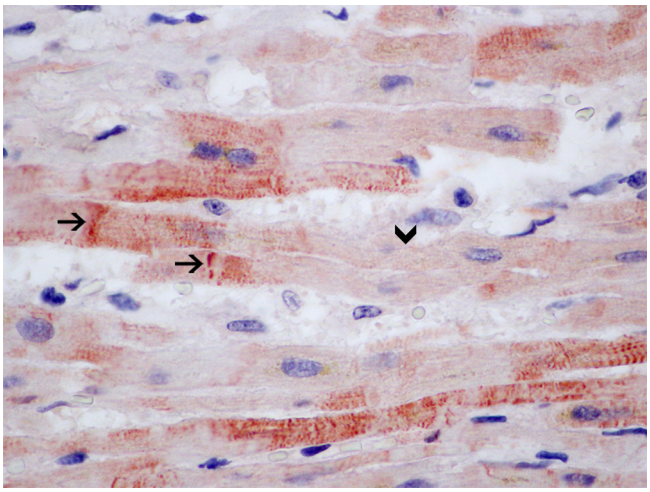

Figure 7 Live-stranded animal: *Delphinus delphis* (reference: i73_13). Intrafibrillar depletion (arrowhead) of cardiac troponin I with intense immunolabelling in the contraction band necrosis (arrows). Immunohistochemical technique: antitroponin I, magnification x 60.

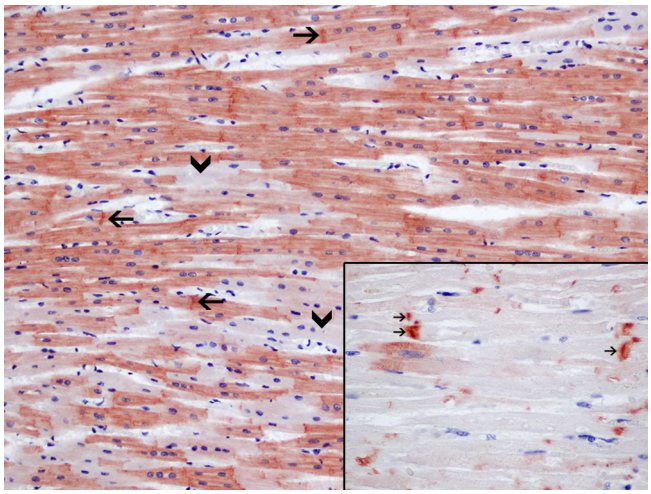

Figure 9 Live-stranded animal: *Delphinus delphis* (reference: i87_03). Intrafibrillar depletion (arrowheads) of cTnC, with intense immunolabelling in the contraction band necrosis (arrows). Immunohistochemical technique: antitroponin C, magnification x 20. Inset: Live-stranded animal: *D. delphis* (reference: i73_13). Detail of the high staining in the contraction band necrosis (arrows) for cTnC. Immunohistochemical technique: antitroponin C, magnification x 60. cTnC, cardiac troponin C.

subepicardial haemorrhages. It is characteristically present in the damaged areas of the myocardium, even after 72 hours.[55] In the present study, the authors detected both oedema and haemorrhages in eight out of the 67 animals (11.9 per cent).

Focal and diffuse subendocardial haemorrhages are visible on gross examination. These conditions are also sometimes detected in the myocardium and epicardium, shortly after large doses of catecholamines are given and/or released in human beings with SCMP.[29 32] In the present study, the authors perceived haemorrhages in 15 animals (22.4 per cent), both macroscopically and microscopically. These findings support previous studies focused on acute deaths associated with stress, particularly in animals that died following live stranding and handling.[39 42 45]

Contraction band necrosis develops within five to 10 minutes of transient ischaemia and reperfusion. It is a characteristic skeletal and myocardial injury associated with the administration of catecholamines, or with high concentrations of endogenous catecholamines.[7 9 27] It is a lesion that is widely described as an indicator of SCMP. This is a condition that is reported in human beings as a consequence of stressful events,[11 23 24 26 27 29–32 56] as well as other animals, such as seals[34] and cetaceans, due to acute deaths following ship collisions, bycatch, live stranding and handling stress.[35 39 40 42 44 45 49] In this study, 33 animals (49.3 per cent of all animals and 100 per cent of animals that died from ship collision, 52.1 per cent of animals that were stranded alive and 8.3 per cent of animals that died from bycatch) presented contraction band necrosis. This condition was more marked at the subendocardial and subepicardial level. Damage was more severe in animals that died from ship collisions, supporting previous studies.[40 57]

The first histological abnormality associated with ischaemia is the presence of long and thinned undulated fibres, termed wavy fibres.[27 55] This abnormality was detected in 29 animals in the present study (43.3 per cent). This lesion is present in animals that had an

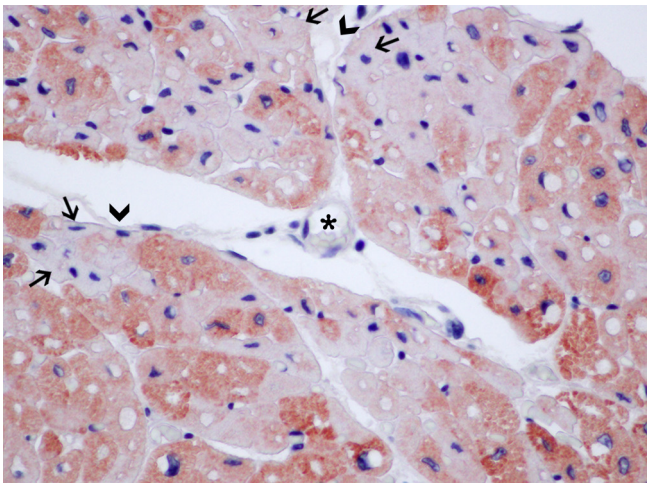

Figure 8 Live-stranded animal: *Delphinus delphis* (reference: i87_03). Degenerated/necrotic cardiomyocytes (arrows) near the blood vessels (*) and interstice (arrow head) present depletion of cardiac troponin C. Immunohistochemical technique: antitroponin C, magnification x 60.

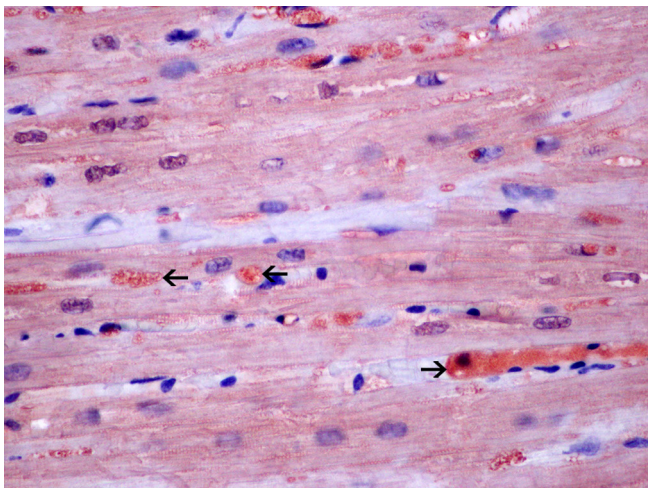

Figure 10 Live-stranded animal: *Steno bredanensis* (reference: i145_04). Myoglobin globules observed inside the blood vessels (arrows) and in the interstitial space, with immunolabelling against myoglobin. Immunohistochemical technique: antimyoglobin, magnification x 60.

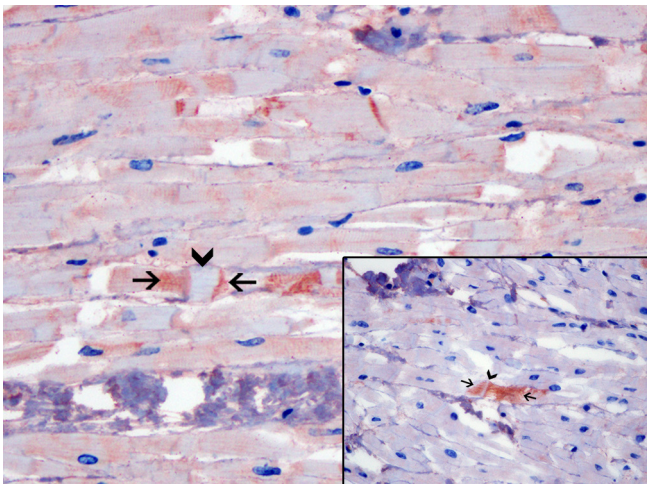

Figure 11 Ship collision animal: *Physeter macrocephalus* (reference: i58_09). Expression of fibrinogen mainly in an area next to (arrows) the contraction band necrosis (arrowhead). Immunohistochemical technique: antifibrinogen, magnification x 40. Inset: Ship collision animal: *P. macrocephalus* (reference: i59_09). Detail of the immunolabelling of fibrinogen in the zone near (arrows) the contraction band necrosis (arrowhead). Immunohistochemical technique: antifibrinogen, magnification x 60.

acute death due to a stressful situation.[35 39 42 49] The wavy fibres are likely to result from stretched fibres that cannot contract, which also occurs in the adjacent myocardium, during the bulging of severely ischaemic tissue that occurs during systole. Thus, this condition could be used as a morphological indicator of early myocardial injury. However, wavy fibres are not specific to necrosis, as they have been observed experimentally shortly after coronary occlusion in the myocardium.[55]

Hypereosinophilia is the first confirmed change specific to myocardial necrosis using histological (HE) and histochemical techniques (PTAH and Masson's trichrome). It consists of an increased eosinophilic, blue or red staining of necrotic cardiomyocytes.[27 55] In

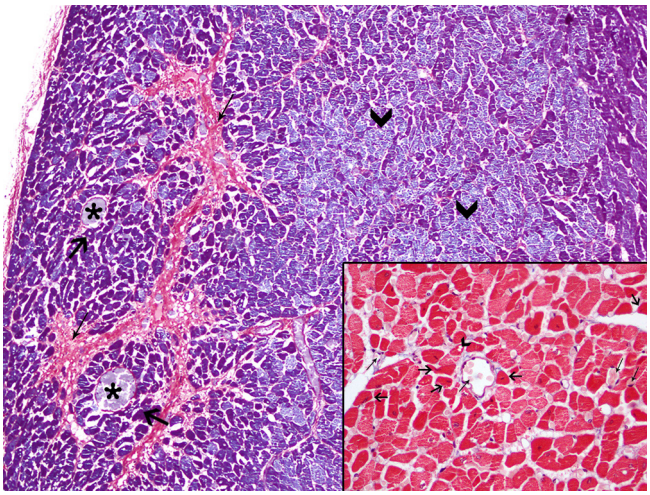

Figure 12 Live-stranded animal: *Delphinus delphis* (reference: i71_07). Groups of damaged cardiomyocytes near the blood vessels (*) which presented higher staining (arrows), in this case due to the PTAH technique, comparing with the normal cardiomyocytes which demonstrate minor staining (arrowheads). Magnification x 10. Take also into consideration the presence of fibrosis (thin arrows). Inset: Live-stranded animal: *Mesoplodon densirostris* (reference: i238_03). Detail of the perivascular pattern through the presence of rows of individual degenerated cells surrounding the blood vessels (thin arrows), with a more intense staining (arrows) with Masson's trichrome technique, magnification x 40. Notice as well the sporadic occurrence of cytoplasmic vacuolisation (arrowhead). PTAH, phosphotungstic acid haematoxylin.

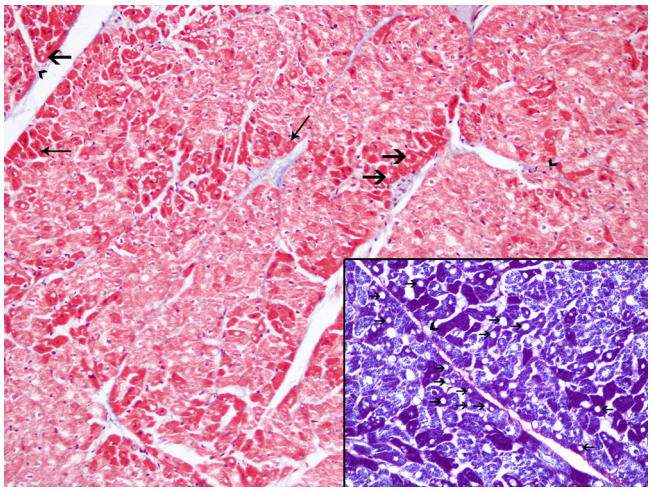

Figure 13 Live-stranded animal: *Delphinus delphis* (reference: i87_03). Groups of damaged cardiomyocytes near the blood vessels (arrowheads) which presented cytoplasmic vacuolisation (arrows). Furthermore, observe the presence of higher staining (thin arrows), in this case due to the Masson's trichrome technique, comparing with the normal cardiomyocytes which demonstrate minor staining. Magnification x 20. Inset: Live-stranded animal: *D. delphis* (reference: i87_03). Detail of the perivascular pattern through the presence of rows of individual degenerated cells surrounding the blood vessels (arrowhead), with vacuolar degeneration (arrows) and a more intense staining with PTAH technique, magnification x 60. PTAH, phosphotungstic acid haematoxylin.

the present study, all animals (100 per cent) exhibited hypereosinophilia. In experimental animals, this colour change and subtle interstitial oedema are evident two to three hours after coronary occlusion, and are more pronounced and more easily recognisable at three to six hours.[55] Animals that die due to a stressful situation present this cytoplasmic change.[35 39 40 42 49] In the present study, hypereosinophilia was more pronounced in the subendocardial and subepicardial zone, supporting previous studies that showed how irreversible injury progresses in a wavefront movement from the ischaemic subendocardium. The perfusion deficit is most severe in the subepicardium, which receives some collateral blood flow.[25 35 49]

Vacuolar degeneration is characterised, morphologically, by the intracellular accumulation of fluid and lysis of myofibrils. It was detected in 65 animals (96.9 per cent) in this study. Cytoplasmic vacuolisation was more noticeable at the subendocardial and subepicardial level in this study. Previous studies commonly detected it in the periphery of myocardial infarctions, and in the subepicardial, subendocardial and perivascular regions which suffer with severe, chronic and fatal ischaemia.[35 49 57] While this condition is quite common, the morphological characteristics and the functional importance of vacuolar degeneration are poorly understood. Even though the pathogenesis is not known, it is hypothesised that it is the result of increased hypoxia induced by myocardial cell membrane permeability, with consequent fluid influx. This phenomenon is normally observed in acute deaths associated with stressful situations.[35 49 57]

Previous studies have demonstrated the necessity of corroborating histological findings with specific markers that might better expose myocardial damage.[39]

Therefore, in this study, the authors compared markers that have been previously used in similar studies, such as myoglobin and fibrinogen,[39 40 42 58 59] with markers used in studies of human heart samples with SCMP, such as cTnI and cTnC.[58 60–62] The leakage of muscle (skeletal and cardiac) proteins begins immediately after vital trauma, which causes an acute ischaemia. As a result, early myocardial cell membrane rupture occurs, causing a quick decline in myoglobin, cTnI and cTnC content, along with the deposition of plasma proteins, such as fibrinogen in cardiomyocytes. Homogeneous labelling for myoglobin was demonstrated in normal cardiac muscle, while injured cardiomyocytes exhibit both the depletion of myoglobin within muscle fibres and the intercellular and interstitial accumulation of myoglobin.[39 40 42 58 59] The current study confirmed the depletion of myoglobin and the accumulation of fibrinogen, particularly in the zone adjacent to and within the contraction band necrosis in the injured cardiomyocytes. Thus, this study is the first to use specific markers, such as cTnI and cTnC, to detect damage to the heart of cetaceans.

Troponin is a regulatory complex of three subunits of proteins released from cardiomyocytes when irreversible myocardial damage occurs. The three subunits are troponin C (calcium binding component), troponin T (binding component tropomyosin) and troponin I (inhibiting component). The subunit cTnI is, at present, the blood test most ordered because it is highly specific for cardiac tissue and provides diagnostic accuracy of myocardial infarction that reflects ischaemia.[63] Although cTnI and cTnC are depleted in damaged cardiomyocytes, as demonstrated in this study, these two markers were strongly expressed in the contraction band necrosis and in some isolated cells showing degeneration. Thus, cells undergoing apoptosis have a greater concentration of troponin due to condensation, while tissue antigens are severely depleted in zones with obvious ischaemic necrosis (infarction zones).[58 60–62]

Recent studies support the concept that pathogenesis is caused by acute myocardial ischaemia. In this study, the authors identified and described damaged cardiomyocytes (ie, individual cells or groups of damaged cells). These cells commonly presented hypereosinophilia and cytoplasmic vacuolisation in a multifocal distribution, particularly in the rows of cardiomyocytes localised in the periphery of blood vessels. These cells followed a perivascular pattern, mainly in live-stranded animals, indicating that the pathogenesis of these lesions was associated with ischaemia-reperfusion. Ischaemia-reperfusion injury is a phenomenon that is described as an accelerated injury to the heart due to blood being resupplied to the ischaemic area of the heart that was previously deprived of its blood supply (ischaemia). As a result, this part of the heart had been subject to hypoxia, substantial loss of cell volume regulation and influx of calcium due to inadequate run of the ion pumps. Membrane damage also occurs when blood supply is re-established to areas with potentially viable cells.[12 25]

Another anatomopathological finding described in the postmortem examination and endomyocardial biopsies of human patients and experimental animals with SCMP is interstitial infiltration of the lymphocytes, leucocytes and macrophages.[24 28 32] In this study, different types of inflammatory cells were identified in 17 animals (25.4 per cent). These cells infiltrated zones with haemorrhages, fibrillary rupture of fibres, degeneration and necrosis of cardiac cells.

Conclusions

To conserve marine mammals appropriately, it is important to understand many aspects of their biology, including the causes and rates of mortality. Mortality in cetaceans arises through both natural events and human activities which may be intentional and unintentional. Examples include live stranding, entanglement in fishing equipment (bycatch) and collisions with boats (ship collision). While stress in cetaceans represents an extreme and multifactorial condition, the presence of vascular changes (congestion, interstitial oedema and haemorrhages), acute degenerative lesions (such as contraction band necrosis, wavy fibres, hypereosinophilia and vacuolar degeneration) and inflammatory infiltration in the heart, following live strandings, capture/rescue interactions, bycatch and ship collisions, demonstrates that cetaceans experience SCMP similar to that of human beings. This is further supported by immunolabelling for biomarkers of early, in vivo, heart damage, like cTnC and cTnI. Therefore, the pathophysiology of SCMP might contribute to mortality following these type of stress events. Consequently, it might compromise subsequent rehabilitation or the effectiveness of therapy and care of recovering animals. In conclusion, this study advances understanding of the pathology of cetaceans that could be used to help decision-making processes during stressful situations and improve conservation efforts by reducing the mortality of these animals.

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Supplementary file. Epidemiologic data, such as, species, sex, age, state of decomposition, stranding location, type of stranding and distinct pathological entity and cause of death, of the 67 selected animals, from the 148 initially examined animals.

NUMBER	ANIMAL CODE	SAMPLES CODE	SPECIES	SEX	AGE	STATE OF DECOMPOSITION	STRANDING LOCATION	TYPE OF STRANDING	PATHOLOGICAL ENTITY / CAUSE OF DEATH
1	CET 097	061/00	*Stenella coeruleoalba*	Male	Undetermined	Very Fresh	Fuerteventura - Canary Islands	Live	Pathology associated with significant loss of nutritional status
2	CET 098	141/00	*Stenella coeruleoalba*	Male	Undetermined	Very Fresh	La Graciosa - Canary Islands	Live	Pathology associated with significant loss of nutritional status
3	CET 110	339/00	*Delphinus delphis*	Male	Adult	Very Fresh	Gran Canaria - Canary Islands	Live	Pathology associated with good nutritional status
4	CET 116	452/00	*Kogia breviceps*	Female	Adult	Fresh	Tenerife - Canary Islands	Live	Pathology associated with significant loss of nutritional status
5	CET 132	i241/01	*Stenella frontalis*	Male	Adult	Very Fresh	La Gomera - Canary Islands	Live	Pathology associated with significant loss of nutritional status
6	CET 134	i245/01	*Mesoplodon europaeus*	Female	Newborn	Very Fresh	Gran Canaria - Canary Islands	Live	Neonatal/Perinatal pathology
7	CET 145	i300/01	*Tursiops truncatus*	Female	Adult	Very Fresh	Gran Canaria - Canary Islands	Live	Massive stranding
8	CET 168	i083/02	*Tursiops truncatus*	Male	Subadult	Fresh	Tenerife - Canary Islands	Dead	Entanglement in fishery lines (Bycatch)
9	CET 170	i087/02	*Stenella coeruleoalba*	Male	Adult	Very Fresh	Fuerteventura - Canary Islands	Live	Pathology associated with good nutritional status
10	CET 177	i108/02	*Stenella frontalis*	Female	Adult	Very Fresh	Gran Canaria - Canary Islands	Live	Pathology associated with significant loss of nutritional status
11	CET 178	i109/02	*Stenella coeruleoalba*	Male	Juvenile	Very Fresh	Gran Canaria - Canary Islands	Live	Pathology associated with good nutritional status
12	CET 206	i087/03	*Delphinus delphis*	Male	Calf	Fresh	Fuerteventura - Canary Islands	Live	Pathology associated with good nutritional status
13	CET 213	i238/03	*Mesoplodon densirostris*	Female	Adult	Fresh	Gran Canaria - Canary Islands	Live	Pathology associated with significant loss of nutritional status
14	CET 229	i015/04	*Stenella frontalis*	Female	Adult	Fresh	Fuerteventura - Canary Islands	Live	Pathology associated with good nutritional status
15	CET 243	i092/04	*Mesoplodon densirostris*	Male	Adult	Very Fresh	Tenerife - Canary Islands	Live	Pathology associated with significant loss of nutritional status
16	CET 260	i130/04	*Stenella coeruleoalba*	Female	Juvenile	Fresh	Fuerteventura - Canary Islands	Live	Pathology associated with good nutritional status
17	CET 261	i132/04	*Stenella frontalis*	Female	Adult	Fresh	Tenerife - Canary Islands	Live	Pathology associated with good nutritional status
18	CET 267	i144/04	*Stenella coeruleoalba*	Male	Adult	Fresh	Gran Canaria - Canary Islands	Live	Pathology associated with good nutritional status

NUMBER	ANIMAL CODE	SAMPLES CODE	SPECIES	SEX	AGE	STATE OF DECOMPOSITION	STRANDING LOCATION	TYPE OF STRANDING	PATHOLOGICAL ENTITY / CAUSE OF DEATH
19	CET 269	i145/04	*Steno bredanensis*	Male	Juvenile	Fresh	Gran Canaria - Canary Islands	Live	Pathology associated with significant loss of nutritional status
20	CET 272	i151/04	*Stenella longirostris*	Male	Adult	Very Fresh	Gran Canaria - Canary Islands	Live	Massive stranding
21	CET 296	i101/05	*Tursiops truncatus*	Female	Subadult	Very Fresh	Gran Canaria - Canary Islands	Live	Pathology associated with good nutritional status
22	CET 305	i225/05	*Tursiops truncatus*	Female	Subadult	Very Fresh	Lanzarote - Canary Islands	Live	Pathology associated with good nutritional status
23	CET 333	i132/06	*Mesoplodon europaeus*	Female	Subadult	Fresh	El Hierro - Canary Islands	Live	Undetermined
24	CET 334	i133/06	*Mesoplodon europaeus*	Female	Subadult	Fresh	El Hierro - Canary Islands	Live	Undetermined
25	CET 339	i145/06	*Globicephala macrorhynchus*	Female	Adult	Fresh	Fuerteventura - Canary Islands	Live	Pathology associated with significant loss of nutritional status
26	CET 360	i262/06	*Globicephala macrorhynchus*	Male	Calf	Very Fresh	Gran Canaria - Canary Islands	Live	Pathology associated with good nutritional status
27	CET 369	i049/07	*Balaenoptera physalus*	Female	Subadult	Moderate Autolysis	Gran Canaria - Canary Islands	Dead	Ship collision
28	CET 370	i052/07	*Stenella coeruleoalba*	Male	Adult	Fresh	Tenerife - Canary Islands	Live	Pathology associated with significant loss of nutritional status
29	CET 373	i071/07	*Delphinus delphis*	Female	Adult	Fresh	La Graciosa - Canary Islands	Live	Pathology associated with significant loss of nutritional status
30	CET 379	i090/07	*Mesoplodon bidens*	Male	Adult	Fresh	Lanzarote - Canary Islands	Dead	Ship collision
31	CET 404	i373/07	*Kogia breviceps*	Male	Adult	Very Fresh	Gran Canaria - Canary Islands	Live	Pathology associated with good nutritional status
32	CET 412	i095/08	*Stenella coeruleoalba*	Male	Calf	Fresh	Gran Canaria - Canary Islands	Live	Pathology associated with good nutritional status
33	CET 413	i102/08	*Pseudorca crassidens*	Male	Juvenile	Fresh	Lanzarote - Canary Islands	Live	Intra- and interspecific traumatic interactions
34	CET 431	i149/08	*Grampus griseus*	Male	Juvenile	Fresh	Tenerife - Canary Islands	Live	Pathology associated with good nutritional status
35	CET 463	i256/08	*Physeter macrocephalus*	Female	Newborn	Very Fresh	La Gomera - Canary Islands	Live	Neonatal/Perinatal pathology
36	CET 473	i343/08	*Steno bredanensis*	Male	Adult	Fresh	Gran Canaria - Canary Islands	Live	Pathology associated with significant loss of nutritional status
37	CET 484	i058/09	*Physeter macrocephalus*	Female	Adult	Fresh	Tenerife - Canary Islands	Dead	Ship collision
38	CET 485	i059/09	*Physeter macrocephalus*	Male	Calf	Fresh	Tenerife - Canary Islands	Dead	Ship collision
39	CET 502	i169/09	*Stenella coeruleoalba*	Female	Subadult	Fresh	Lanzarote - Canary Islands	Live	Pathology associated with good nutritional status
40	CET 534	i136/10	*Grampus griseus*	Male	Subadult	Fresh	Tenerife - Canary Islands	Live	Pathology associated with significant loss of nutritional status

NUMBER	ANIMAL CODE	SAMPLES CODE	SPECIES	SEX	AGE	STATE OF DECOMPOSITION	STRANDING LOCATION	TYPE OF STRANDING	PATHOLOGICAL ENTITY / CAUSE OF DEATH
41	CET 574	i145/11	*Stenella coeruleoalba*	Male	Adult	Fresh	Gran Canaria - Canary Islands	Live	Pathology associated with significant loss of nutritional status
42	CET 577	i171/11	*Stenella coeruleoalba*	Male	Adult	Fresh	Tenerife - Canary Islands	Live	Pathology associated with good nutritional status
43	CET 578	i183/11	*Grampus griseus*	Female	Adult	Fresh	La Gomera - Canary Islands	Live	Pathology associated with significant loss of nutritional status
44	CET 583	i229/11	*Globicephala macrorhynchus*	Male	Juvenile	Fresh	Tenerife - Canary Islands	Live	Pathology associated with significant loss of nutritional status
45	ALCET11006	i249/11	*Tursiops truncatus*	Female	Calf	Moderate Autolysis	Almería - Andalucía	Dead	Entanglement in fishery lines (Bycatch)
46	MACET11042	i256/11	*Delphinus delphis*	Female	Calf	Fresh	Málaga - Andalucía	Dead	Entanglement in fishery lines (Bycatch)
47	CACET11010	i263/11	*Delphinus delphis*	Female	Juvenile	Fresh	Cádiz - Andalucía	Dead	Entanglement in fishery lines (Bycatch)
48	CACET11025	i264/11	*Delphinus delphis*	Male	Newborn	Moderate Autolysis	Cádiz - Andalucía	Dead	Entanglement in fishery lines (Bycatch)
49	CACET11037	i267/11	Delphinus delphis	Male	Calf	Moderate Autolysis	Cádiz - Andalucía	Dead	Entanglement in fishery lines (Bycatch)
50	CET 627	i099/12	*Kogia breviceps*	Female	Calf	Very Fresh	Tenerife - Canary Islands	Live	Neonatal/Perinatal pathology
51	DDE020812CAM	i106/12	Delphinus delphis	Male	Juvenile	Moderate Autolysis	Cádiz - Andalucía	Dead	Entanglement in fishery lines (Bycatch)
52	TTR110612MAV	i113/12	*Tursiops truncatus*	Female	Adult	Very Fresh	Málaga - Andalucía	Live	Entanglement in fishery lines (Bycatch)
53	CET 636	i148/12	*Mesoplodon mirus*	Male	Subadult	Fresh	El Hierro - Canary Islands	Dead	Ship collision / Intra- and interspecific traumatic interactions
54	CET 642	i013/13	*Stenella frontalis*	Female	Adult	Fresh	Lanzarote - Canary Islands	Live	Pathology associated with good nutritional status
55	CET 655	i048/13	*Balaenoptera acutorostrata*	Male	Calf	Fresh	Fuerteventura - Canary Islands	Dead	Entanglement in fishery lines (Bycatch)
56	CET 663	i062/13	*Delphinus delphis*	Female	Adult	Fresh	Tenerife - Canary Islands	Live	Pathology associated with good nutritional status
57	CET 668	i073/13	*Delphinus delphis*	Female	Adult	Fresh	Tenerife - Canary Islands	Live	Pathology associated with significant loss of nutritional status
58	DDE270813MAM1	i229/13	*Delphinus delphis*	Female	Adult	Fresh	Málaga - Andalucía	Dead	Entanglement in fishery lines (Bycatch)
59	SCO310813HUM	i235/13	*Stenella coeruleoalba*	Female	Adult	Fresh	Huelva - Andalucía	Dead	Entanglement in fishery lines (Bycatch)
60	CET 717	i151/14	*Stenella coeruleoalba*	Male	Subadult	Very Fresh	Gran Canaria - Canary Islands	Live	Pathology associated with significant loss of nutritional status
61	CET 718	i155/14	*Physeter macrocephalus*	Female	Adult	Fresh	Tenerife - Canary Islands	Dead	Ship collision
62	CET 725	i225/14	*Stenella frontalis*	Male	Adult	Fresh	Tenerife - Canary Islands	Live	Pathology associated with good nutritional status
63	CET 748	i236/15	*Stenella coeruleoalba*	Male	Subadult	Fresh	Lanzarote - Canary Islands	Live	Pathology associated with good nutritional status

NUMBER	ANIMAL CODE	SAMPLES CODE	SPECIES	SEX	AGE	STATE OF DECOMPOSITION	STRANDING LOCATION	TYPE OF STRANDING	PATHOLOGICAL ENTITY / CAUSE OF DEATH
64	SCO110314CAM	i288/14	Stenella coeruleoalba	Male	Juvenile	Moderate Autolysis	Cádiz - Andalucía	Dead	Entanglement in fishery lines (Bycatch)
65	CET 771	i414/15	*Ziphius cavirostris*	Female	Adult	Fresh	Tenerife - Canary Islands	Dead	Ship collision
66	CET 776	i461/15	*Tursiops truncatus*	Male	Juvenile	Fresh	Tenerife - Canary Islands	Live	Pathology associated with good nutritional status
67	CET 819	i950/16	*Balaenoptera edeni*	Male	Calf	Very Fresh	Fuerteventura - Canary Islands	Live	Neonatal/Perinatal pathology

6.2 CORRELATION OF THE BLOOD VALUES DETECTED FOR THE BIOCHEMICAL MARKERS OF ACUTE CARDIAC MUSCLE DAMAGE WITH THE PATHOLOGICAL FINDINGS IDENTIFIED HISTOLOGICALLY

The second specific objective of this work, which consisted in correlating the blood values detected for the biochemical markers of acute cardiac muscle damage with the pathological findings identified at a microscopic level, was achieved using different phases as mentioned in the materials and methods chapter.

In the first phase, we estimated a baseline range for cTnI in cetaceans, since there is not an existing one in the current literature, through the measurements obtained from captive animals.

The second phase consisted in comparing the values from 9 live-stranded cetaceans with the normal baseline range of cetaceans (determined previously by us) and also with other mammals, such as humans and dogs.

In the third and fourth phase, we used histological, histochemical and immunohistochemical approaches to evaluate the acute cardiac lesions at a microscopic level.

Finally, we correlated, using the available literature on the biochemical kinetics and the chronological sequence of an acute ischemic injury in the heart in the histology, our blood results with the histopathological lesions in our animals.

All of this resulted in the publication of an article, as you can see subsequently, titled ***Plasma cardiac troponin I in live-stranded cetaceans: correlation with pathological findings of acute cardiac injury***, on January 2020 in the *Scientific Reports* journal, Volume 10, Issue 1 (doi: 10.1038/s41598-020-58497-3), having this journal an impact factor of 4.011 in 2018 and therefore being a Q1 in the veterinary category.

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Increased Plasma Cardiac Troponin I in Live-Stranded Cetaceans: Correlation with Pathological Findings of Acute Cardiac Injury

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Capture myopathy (CM), is a syndrome that occurs as the result of the stress during and after capture, handling, restraint, and transport of wild animals. Although CM has been described for many species of cetaceans, characterization of the acute cardiac injury - an important component of this syndrome - are still scarce. In this study, we firstly estimated a normal range for cardiac troponin I (cTnI) on cetaceans. Here, through biochemical analysis (especially of cTnI) and histopathological, histochemical, and immunohistochemical correlations with decreased troponin immunolabelling, we studied the cardiac injury in live-stranded cetaceans. Nine cetaceans which stranded alive on the Canary Islands (January 2016 - June 2019) were included in this study. Sampled individuals presented elevated values of plasma cTnI, which were correlated to histopathological lesions comprised of vascular changes and acute degenerative lesions. Immunohistochemically, injured cardiomyocytes showed a decreased intrafibrillar troponin immunoreaction. This is the first attempt to establish a normal baseline range for cTnI in cetaceans, and the first study comparing plasma biomarkers values with histopathological and immunohistochemical findings. This approach allowed us to demonstrate the degree of cardiac damage as a result of injury, consistent with ischemia–reperfusion lesions. The knowledge gained here could improve decision-making procedures during stressful situations, mainly in live-strandings, handling, and rehabilitation, thereby reducing the mortality of cetaceans.

All life forms have evolved mechanisms to cope with stressful situations in their lives[1]. For cetaceans, live-stranding is a situation in which they are alive on the beach or in shallow water, and in distress due to being unable to free themselves and resume normal activity[2–4]. Similar to humans and other animals, once the central nervous system of a cetacean perceives an internal (physiologic or psychogenic) or environmental threat to their homeostasis, the threat being the stressor, a biological response is developed[5–7].

Irrespective of the animal's previous health, stranding creates an anomalous and extreme situation for an organism that it is not anatomically or physiologically adapted to handle. It is a pathological entity in which acute stress is the central axis of its etiopathogenesis, presenting clinical and lesional findings that can cause the death of the animal or seriously aggravate a previous disease over the period of capture or captivity. This reaction may in turn influence the subsequent rehabilitation and recovery of affected animals, since live-stranded cetaceans are frequently debilitated when rescued. Therefore, although the stranding response is intended to improving the health and welfare of these animals, some "rescue and recovery" activities may in fact be counterproductive[4,8–13].

Deaths of live-stranded cetaceans may be attributed to the so-called "stress response syndrome" or "alarm reaction," which are thought to have analogous mechanisms to capture myopathy (CM), in which cardiac damage due to the extreme stress seems to play an important role[2,8,10–12,14,15]. For this reason, previous authors have suggested that cetaceans would be especially predisposed to develop stress cardiomyopathy (SCMP), comparable to SCMP in humans which appears to be related to an excess of plasma catecholamines triggered by a stressful

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event[2,8,10–12,14–27]. There are no previous studies regarding biochemical cardiac markers that indicate damage to the heart of marine mammals (more specifically in cetaceans).

When irreversible cardiac damage occurs, troponin is released from cardiomyocytes. Troponin is a regulatory protein complex comprised three subunits; troponin C, the calcium binding component; troponin T, the binding component tropomyosin; and troponin I, the inhibitory component. All components are involved in the contractile process of both skeletal and cardiac muscle. The troponin C expressed in skeletal muscle is identical to cardiac troponin C (cTnC). However, cardiac troponin T (cTnT) and cardiac troponin I (cTnI) are specific to the heart[28].

Very low amounts of cTnI are detectable in the blood of healthy human individuals with no evidence of cardiac disease, as opposed to the amount of cardiac troponin released from cardiomyocytes into the blood as a result of injury, which is consistent with ischemia and other causes[29–31]. Due to this specificity, cTnT and cTnI are recommended by various international societies as a diagnostic indicator for acute myocardial infarction (AMI) and other heart pathologies, such as SCMP, since they are potential markers of cardiac damage[32].

The histology that defines CM consists of ischemia-reperfusion injuries consistent with local to generalised vasospasm and vasodilation (catecholamines, neurogenic shock, and impeded venous flow return from body compression, causing hypoxia in different organs), direct traumatic injury to the muscle resulting in acute to subacute degeneration (rhabdomyolysis), acute renal failure associated with myoglobinuric nephrosis, and areas of necrosis in viscera[9–11,13–15,33–35].

The aim of this study was to estimate a normal range for cTnI in cetaceans and describe the acute cardiac injury in live-stranded cetaceans, correlating biochemical analysis with histological, histochemical, and immunohistochemical findings.

Results

The reference range of cTnI was of 0–0.0256 µg/L, determined with a 95% probability. All data necessary to calculate this range are shown in Supplemental Table 1.

The serum values of cTnI and creatine kinase (CK) obtained for each animal are shown in Table 1. Additionally, it summarises the evaluation of the vascular changes, acute degenerative changes, and the presence of inflammatory cells. It also shows the presence or absence of myoglobin globules.

Regarding animal 1, the biochemical analysis presented 0.06 µg/L for cTnI and 1875.0 U/L for CK. With reference to the histopathological results, we observed a moderate epicarditis, neuritis, and multifocal lymphoplasmacytic perineuritis, and a mild multifocal lymphoplasmacytic myocarditis. We also detected moderate multifocal myocardial fibrosis and mild multifocal acute degenerative changes in the cardiomyocytes consistent with wavy fibres, hypereosinophilia, and cytoplasmic vacuolisation (Fig. 1). We did not assess the depletion or expression of the immunohistochemical markers because the sample was frozen, and previous studies have shown that animals with advanced autolysis or that were previously frozen presented a greater number of false negatives when using histochemical and immunohistochemical techniques[15].

Biochemically, due to serum insufficiency, the only metric we were able to obtain from animal 2, was a cTnI level of 40.00 µg/L. The histopathology revealed a mild, multifocal congestion and haemorrhages. Moreover, it also showed moderate, multifocal acute cardiomyodegenerative lesions indicated as hypereosinophilia and cytoplasmic vacuolisation. Immunohistochemically, we verified homogenous, intrafibrillar depletion of cTnI, cTnC, and myoglobin in the degenerated/necrotic cardiomyocytes. Furthermore, damaged cells from the cardiac muscle, showed various intensities of immunolabeling for fibrinogen.

The biochemical analysis of animal 3, it presented 0.025 µg/L of cTnI and 1667.1 U/L of CK. We were incapable to obtain histopathological and immunohistochemical parameters, as we were unable to perform a necropsy on this animal, since it was used for anatomical research purposes.

Biochemically, due to serum insufficiency, the only metric we were able to obtain from animal 4, was a cTnI level of 0.235 µg/L. The histopathology revealed mild to moderate, multifocal congestion, and interstitial oedema. Additionally, we observed moderate to severe multifocal acute degenerative changes in the cardiomyocytes, such as hypereosinophilia and cytoplasmic vacuolisation. Immunohistochemically, we detected fibrinogen expression and the disappearance of immunolabeling for cTnI, cTnC, and myoglobin in the degenerated/necrotic cardiomyocytes.

Regarding animal 5, the biochemical analysis showed a cTnI level of 0.249 µg/L and a CK level of 3521.2 U/L. The histopathology revealed mild to moderate acute cardiomyodegenerative lesions, consistent with contraction band necrosis (Fig. 2), wavy fibres, hypereosinophilia, and cytoplasmic vacuolisation. Simultaneously, we also verified myoglobin globules and mild, multifocal congestion, and interstitial oedema. The immunohistochemical analysis showed the homogenous intrafibrillar depletion of cTnI, cTnC, and myoglobin in the degenerated/necrotic cardiomyocytes; these also exhibited various intensities of immunolabeling for fibrinogen.

The biochemical results for animal 6 showed 0.748 µg/L for cTnI and 3299.5 U/L for CK. The histopathology showed moderate multifocal myocardial fibrosis and acute degenerative changes in the cardiomyocytes, reflected as wavy fibres, hypereosinophilia, and cytoplasmic vacuolisation. Furthermore, we detected the presence of myoglobin globules and mild multifocal congestion. The immunohistochemical parameters showed the expression of fibrinogen and the disappearance of immunolabeling for cTnI, cTnC, and myoglobin in the degenerated/necrotic cardiomyocytes.

Biochemically animal 7 showed 0.033 µg/L for cTnI and 383.6 U/L for CK. The histopathology showed mild acute cardiomyodegenerative lesions, such as hypereosinophilia and cytoplasmic vacuolisation. We did not analyse depletion or expression of immunohistochemical marker because the sample was frozen.

For animal 8, serum insufficiency prevented us from obtaining all biochemical metrics. We were only able to obtain the cTnI level, which was 0.06 µg/L. We were unable perform histopathology and immunohistochemistry because we were unable to perform a necropsy on this animal as it was released back into the sea.

			Animal 1 (*)	Animal 2 (†)	Animal 3 (‡)	Animal 4 (§)	Animal 5 (‡)	Animal 6 (§)	Animal 7 (‡)	Animal 8 (‖)	Animal 9 (*)
			Delphinus delphis	*Delphinus delphis*	*Stenella coeruleoalba*	*Stenella coeruleoalba*	*Globicephala macrorhynchus*	*Delphinus delphis*	*Stenella coeruleoalba*	*Stenella coeruleoalba*	*Stenella coeruleoalba*
STRANDING CIRCUNSTANCES	TIME OF STRANDING NOTICE		10:16 AM	11:25 AM	5:12 PM	11:13 AM	11:57 AM	9:30 AM	8:37 AM	9:38 AM	12:35 PM
	TIME OF DEATH		12:00 PM	12:00 PM	6:45 PM	NO	12:00 PM	NO	8:45 AM	NO	2:38 PM
	TIME OF SAMPLE COLLECTION		10:00 PM	4:00 PM	6:45 PM	11:47 AM	3:37 PM	10:00 AM next day	11:00 AM	2:00 PM	3:28 PM
BIOCHEMICAL ANALYSIS	TROPONIN I (cTnI) (µg/L)	*Animal Value*	0.06	40.00	0.025	0.235	0.249	0.748	0.033	0.06	0.049
		Baseline Range Cetaceans	0–0.0256 (#)								
		Baseline Range Dogs	≤0.03–0.07 (**)								
		Baseline Range Humans	≤0.1 (**)								
	CREATININE KINASE (CK) (U/L)	*Animal Value*	1875.0	Not measured	1667.1	Not measured	3521.2	3299.5	383.6	Not measured	739.2
		Baseline Range	100–250 (††)								
HISTOPATHOLOGICAL & HISTOCHEMICAL ANALYSIS	VASCULAR CHANGES	*Congestion*	Absent	Mild	No necropsy	Mild to moderate	Mild	Mild	Absent	No necropsy	Mild
		Interstitial Oedema	Absent	Absent		Mild to moderate	Mild	Absent	Absent		Absent
		Haemorrhages	Absent	Mild		Absent	Absent	Absent	Absent		Absent
	ACUTE DEGENERATIVE CHANGES	*Contraction Band Necrosis*	Absent	Absent		Absent	Mild to moderate	Absent	Absent		Absent
		Wavy Fibers	Mild	Absent		Absent	Mild to moderate	Moderate	Absent		Absent
		Hypereosinophilia	Mild	Moderate		Moderate to severe	Mild to moderate	Moderate	Mild		Moderate
		Cytoplasmic Vacuolization	Mild	Moderate		Moderate to severe	Mild to moderate	Moderate	Mild		Moderate
	INFLAMMATORY INFILTRATION		Moderate	Absent		Absent	Absent	Absent	Absent		Absent
	MYOGLOBIN GLOBULES		Absent	Absent		Absent	Present	Present	Absent		Absent
IMMUNOHISTO-CHEMICAL ANALYSIS	MYOGLOBIN		Frozen animal	Depletion	No necropsy	Depletion	Depletion	Depletion	Frozen animal	No necropsy	Depletion
	CARDIAC TROPONIN I (cTnI)			Depletion		Depletion	Depletion	Depletion			Depletion
	CARDIAC TROPONIN C (cTnC)			Depletion		Depletion	Depletion	Depletion			Depletion
	FIBRINOGEN			Deposition		Deposition	Deposition	Deposition			Deposition

Table 1. Summary of the stranding circumstances, biochemical results, histopathological findings and immunohistochemical changes for each animal of the study. (*) Live stranded animal that dies during transport. (†) Live stranding notification (09:00 PM) of a cetacean swimming very close to shore being reintroduced in to sea several times by general public until he swims away. New communication of stranded animal one day and a half after the first notification (11:25). The animal is euthanized. (‡) Live stranded animal that died before being attended. (§) Dead animal which presented injuries that indicates that the animal stranded alive. (‖) Live stranded animal and released back into the sea. (#) Baseline range determined in this study. Comparing the interval of reference for cTnI in bottlenose dolphins with the normal values for humans and dogs we can conclude that is shorter than in other species. (**) Values from literature[41–43]. (††) Reference range for Tursiops truncatus in captivity[4].

Biochemically, animal 9 exhibited 0.049 µg/dL for cTnI and 739.2 U/L for CK. The histopathology showed a mild multifocal congestion and moderate, multifocal acute degenerative changes in the cardiomyocytes, such as hypereosinophilia and cytoplasmic vacuolisation. Regarding the immunohistochemistry, we observed homogenous intrafibrillar depletion of cTnI, cTnC, and myoglobin in the degenerated/necrotic cardiomyocytes; we also detected various intensities of immunolabeling for fibrinogen.

Discussion

Recent studies have characterized cardiac lesions associated with live-stranding in cetaceans. The etiopathogenesis of this acute cardiac pathology has as its central axis the stress derived from stranding, handing, interaction with humans, transport and captivity (having been compared with the CM described in terrestrial mammals). Etiological and lesionally, the acute cardiomyopathy associated with live-stranding is comparable to SCMP in humans. SCMP lesions in live-stranded cetaceans consist of vascular changes (congestion, interstitial edema and hemorrhages) and acute necrotic degenerative lesions (contraction band necrosis, wavy fibers, hypereosinophilia and cytoplasm vacuolization) of perivascular distribution, indicative of ischemia-reperfusion damage[10–12,15,35].

In humans, the diagnostic criteria for SCMP comprises alterations in medical exams (such as electrocardiography, echocardiography, cardiac catheterizations), and biochemical analysis. The more common laboratories abnormalities are consistent with a small, rapid rise to above the normal levels of cTnI and/or CK[36–38].

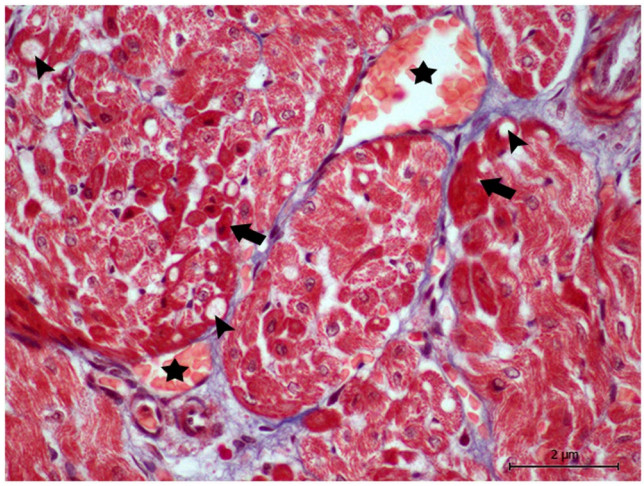

Figure 1. Animal 4. Groups of damaged cardiomyocytes near the blood vessels (*) present a higher staining (arrows), in this case due to Masson's trichrome, compared to normal cardiomyocytes, which stain less. Note the cytoplasmic vacuolisation (arrow heads). Magnification 60 × .

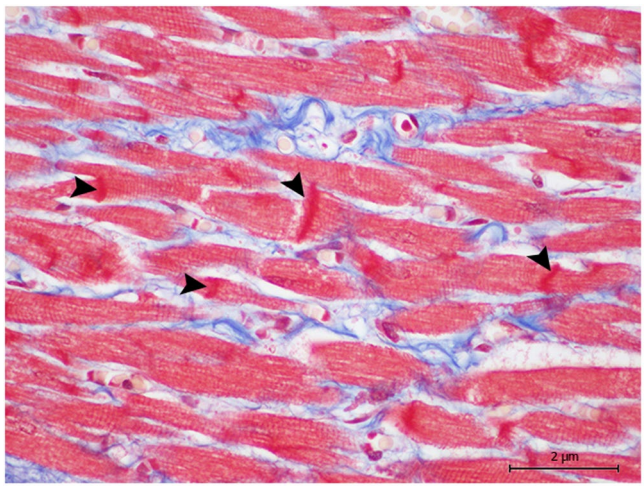

Figure 2. Animal 5. The contraction band necrosis runs (arrow heads) transversely through the cardiomyocytes and is identified through the higher colour intensity with Masson's trichrome. Magnification 60 × .

Currently, there are no hematological and/or biochemical studies on cetaceans that analyze specific markers to detect acute heart damage associated with the live-stranding.

The most commonly used serum enzyme in the determination of neuromuscular damage in animals is CK; it is also used in the detection of myocardial injury in humans[30,39]. CK rises less than 3–12 h after the muscular injury, spiking within 12–24 h and returning to baseline after 48–72 h, unless a new injury or permanent damage has occurred[30]. As verified by the present study, and in comparison with the publish literature, CK values were high for all animals we were able to measure it (animals 1, 3, 5, 6, 7, and 9).

However, although CK is considered a sensitive marker of myocardial damage, it is also present in the skeletal muscles in high concentrations, as well as in the intestine, diaphragm, uterus, and prostate in minor amounts; thus, it has poor specificity when used to detect heart damage[40]. Consequently, troponins have been adopted as the new gold standard in human pathology, since cTnI is detectable in very low quantities (for example 0.01 µg/L) in the blood of healthy individuals with no evidence of cardiac disease[29–32]. Therefore, it is thought that significant elevations (≥0.1 µg/L) of this marker most likely reflect myocardial necrosis, and has been billed as cardiospecific by some authors due to its myocardial tissue specificity, as well as its high sensitivity[30–32]. For this reason, cTnI is used to detect several heart pathologies, such as AMI and SCMP[30,36,40].

Clinical-pathological data evaluation was challenging because values from different species of cetaceans are scarce. Considering this, and because there is no normal range for cTnI in cetaceans to date, we decided that it was important to determine a range of baseline values in order to compare the normal interval for the bottlenose dolphin with the normal parameters of other species, such as humans and dogs, as well as to assess the results of the live-stranded animals in this study.

Comparing the range in this particular study (0–0.0256 µg/L) with the normal values for humans (≤ 0.1 µg/L) and dogs (≤ 0.03–0.07 µg/L), we can conclude that the interval of reference for cTnI in bottlenose dolphins is shorter than in other species[41–43]. This could be justified by the small number of samples (20 blood samples in total). Nonetheless, we consider this result to be an important contribution to clinical biochemistry in cetaceans, as it may help in decision-making and treatment procedures during stressful situations, such as live-strandings, and improve conservation efforts by reducing the mortality of these animals.

Our results, from the live-stranded cetaceans, showed that one of the animals presented a cTnI value within normal range whilst the remaining eight animals demonstrated an increase in comparison to the normal/baseline value for cTnI (0–0.0256 µg/L) that we obtained from captive bottlenose dolphins. However, when comparing these with those for humans (≤ 0.1 µg/L) and dogs (≤ 0.03–0.07 µg/L), we observed that animal 1 (0.06 µg/L), animal 3 (0.025 µg/L), animal 7 (0.033 µg/L), animal 8 (0.06 µg/L), and animal 9 (0.049) are within range, while animal 2 (40.00 µg/L), animal 4 (0.235 µg/L), animal 5 (0.249 µg/L), and animal 6 (0.748 µg/L) exceed the upper value[41–43].

More than 90% of patients with SCMP revealed raised cTnI when measured by conventional assays[36]. However, SCMP is a disease with a high rate of misdiagnosis because it is an important differential diagnosis of an AMI since highly similar clinical presentations occur between them[25,26]. Besides, it is under-recognized because of the unnecessary delay in the diagnosis process in post-mortem examination cases of AMI caused by the less sensitive conventional markers.

Previous authors have stated the necessity of establishing diagnostic biochemical cardiac markers for the post-mortem diagnosis of AMI and SCMP due to the limitations of histopathology. The sensitivity of cTnI makes early detection of microinfarction possible after the onset of ischemia using a rapid one-step assay in body fluids in autopsy cases[40]. Therefore, we selected cTnI as our marker of choice to detect myocardial damage in live-stranded cetaceans, through biochemical and immunohistochemical detection, on the grounds of its specificity, as well as being highly sensitive[30,40].

Generally, injured cardiac myocytes release troponin 3–9 h after ischemic damage, peaking after 12–48 h and levels remain elevated for 4–7 days for cTnI[30,40,44]. Early recognition of myocardial necrosis (1–3 h) is not possible by monitoring kinetics, and these markers are ineffective until 6 or more hours after the onset of the AMI and/or SCMP. The precise determination of the timing of the stress event and/or symptom onset is often exceptionally difficult because it is focused around the clinical patient report. Therefore, in humans, a precondition to obtaining a satisfactory ability to distinguish these pathologies is that blood should be collected 6–9 h after onset[40]. In the case of a live-stranding event, it is also often clinically and pathologically challenging to have the correct timing of the stressful episode, since we are working with wild animals, stranding networks, and the general public. When notified of stranding it is important to acknowledge that the animal could have been stranded recently or could have been stranded for hours before being detected.

Moreover, previous studies have proposed that measuring the level of troponin in the serum can be an important auxiliary method of examining sudden death, since its peak concentration can be related to the degree of injury[40]. For this reason, this finding was confirmed with the histological, histochemical, and immunohistochemical analyses.

An acute ischemic injury, such as the one that occurs in the AMI or SCMP, is determined by morphological alterations, consisting of vascular changes and acute degenerative lesions based on the histological analysis of myocardial tissue obtained from biopsied and post-mortem examined species, mostly humans, but also in cetaceans[15,19,25,26,37,45–54].

In acute ischemic injuries there is a chronological sequence of changes, which can be observed as early as the first 5 minutes (presence of long, thinned, wavy fibers separated by spaces representing edema, and microvascular congestion, at borders of ischemic myocardium). In the following 15 minutes cell death may start to occur. Early changes of cardiomyocyte coagulation necrosis with nuclear pyknosis, color change, more specifically "brick red change" or cytoplasm hypereosinophilia, focal contraction bands and subtle interstitial edema are evident within 2–3 h. Hypereosinophilia and edema become more pronounced and more easily recognizable after a period of 3–6 h. Subsequently, 6–12 h later, an increased number of neutrophils line up in capillaries as well as accelerated changes and more extensive contraction band necrosis with reperfusion are noticeable. In the next 12-hour period, extravasation into interstitial space of neutrophils happens. Vascular congestion, interstitial edema, and focal areas of haemorrhage are also recognized. Thereafter, the subacute period starts[15,26].

Considering all the above, we conclude that all the results obtained from the animals of this study were in accordance with the biochemical kinetics, as well as the chronological sequence of histopathological changes in an acute ischemic injury.

Animal 1 died 2 h after the stranding notice and histopathologically presented mild acute degenerative changes, which indicate that they would fit into the 0–3 h time frame, as expected following the histological sequence.

Elevated biomarker values were observed in animal 2. This can be explained by the fact that the notification, of a cetacean swimming very close to shore, was received and after several attempts by members of the public to reintroduce it into the ocean the animal swam away; despite all the effort 38 h later a new alert was received stating that the animal stranded again. Its histopathology analysis showed mild vascular changes and moderate acute cardiomyodegenerative lesions. All of these are detectable between 0–24 h approximately and in agreement with both biochemical kinetics and histopathological sequence previously mentioned.

The biochemical results for animal 3 were near the maximum of the normal range value for cTnI in cetaceans. The animal died 1 h after the stranding notice, nonetheless, we were unable to compare these results with the histopathological lesions since a necropsy could not be performed.

The external injuries in animal 4 indicated that it had stranded alive, but was already dead by the time it was detected at the beach. Although the time of death is unknown, cTnI value was elevated. Histologically, it presented

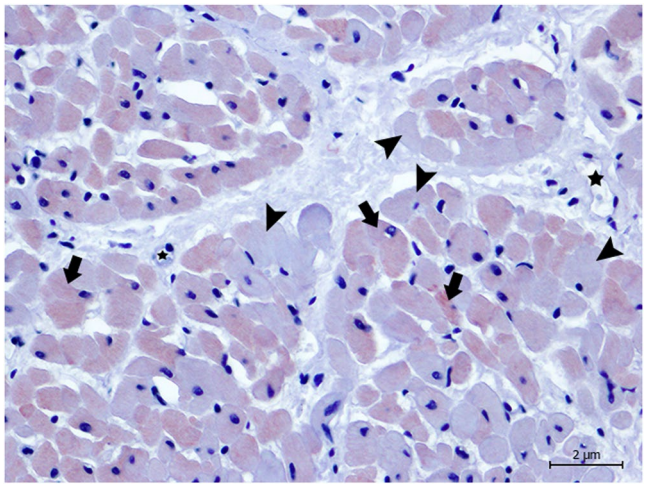

Figure 3. Animal 5. Degenerated/necrotic cardiomyocytes (arrow heads) near the blood vessels (*) present the depletion of cTnI when compared with normal cardiomyocytes (arrows). Magnification 40 ×.

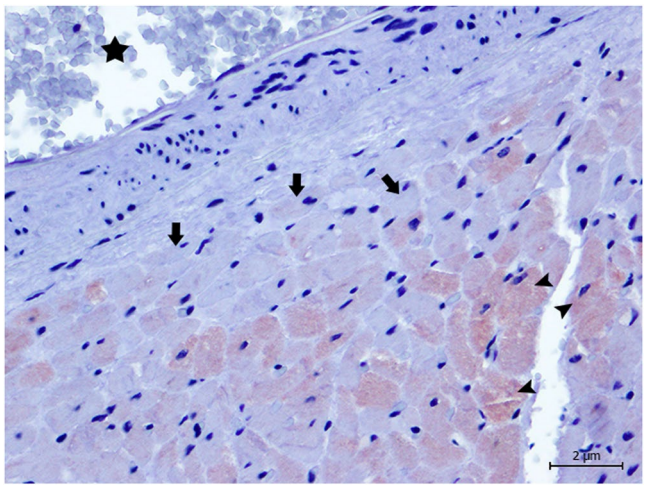

Figure 4. Animal 6. Damaged cardiomyocytes (arrows) reveal a perivascular pattern (*) with decreased immunolabeling for cTnI, in comparison to normal cells (arrow heads). Magnification 40 ×.

mild to moderate vascular alterations, and moderate to severe acute degenerative changes. This fact was consistent, with the time frame of 0–24 h, as per the histological sequence discussed earlier.

Considering the biochemical kinetics in animal 5, which died 1 h after the notice, we hypothesise that the stressful event took place sometime before we received the communication of the stranding, since the cTnI results were high. The mild vascular changes, and mild to moderate acute cardiomyodegenerative lesions were compatible with the time frame of 0–24 h of the histological sequence.

In spite of the unknown time of death of animal 6, and similarly to animal 4, the cTnI results were high and revealed mild vascular alterations and moderate degree of acute degenerative changes, which were comparable to the ones illustrated in the 0–24 h time frame of the histological sequence described previously.

The biochemical values obtained for animal 7 are consistent with the kinetics, since the animal died minutes after the stranding notice. Histopathologically presented mild acute cardiomyodegenerative lesions complying with the time frame of 0–3 h expectations for an acute ischemic injury sequence.

Regarding animal 8, the values challenged the kinetics, because the blood sample was collected 5 hours after the stranding event. However, as discussed previously, the levels of cTnI increases 3–9 h after the stressful event. In view of the fact that we were able to release the animal back to the sea, no histopathological study could be carried out for this animal.

Finally, the results of animal 9 are in accordance with the biokinetics mentioned above, since the animal died 2 h after notification of the stranding. It also presented a mild degree of vascular alterations and moderate acute degenerative changes, common to the sequence of an acute ischemic injury histology, for 0–3 h time frame.

It is important to highlight that active stranding implies a bias in the cases analysed. This is because most animals tend to strand themselves due to a previous pathological process, which is not always identifiable. Therefore,

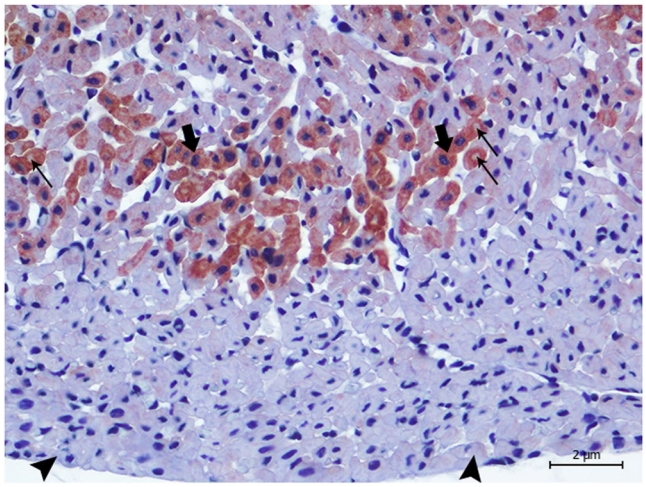

Figure 5. Animal 2. Degenerated/necrotic cardiomyocytes (arrow heads), especially in the epicardium, present the depletion of cTnC when compared to the normal cardiomyocytes (arrows). Note the presence of cytoplasmic vacuolisation (thin arrows). Magnification 40 ×.

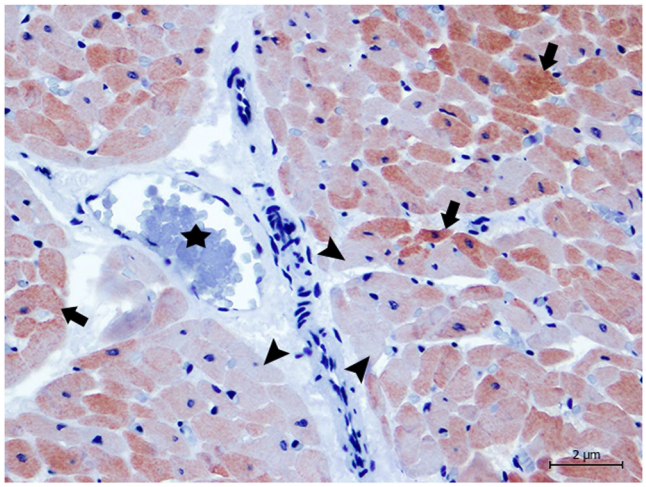

Figure 6. Animal 5. Damaged cardiomyocytes (arrow heads) reveal a perivascular pattern (*) with decreased immunolabeling for cTnC, in comparison to normal cells (arrows). Magnification 40 ×.

the histological lesions described previously were, most likely, directly related to the stranding itself but not related to the cause of the stranding. Nevertheless, the live-stranding could negatively influence the pre-existing pathological process in the animal, aggravating it, and contributing to its death.

For example, animal 1 became stranded/died as a result of a *Morbillivirus* infection (unpublished data) and demonstrated characteristic lesions, such as lymphoplasmacytic infiltration, which can be observed in both *Morbillivirus* infection and in an acute ischemic injury such as the one resulting in AMI or SCMP[8,9,19,45,46]. Another example is the presence of fibrosis, both in animal 1 and animal 6, indicating a chronic phase of the cardiomyocytes damage, since connective tissue proliferation dominates the third week until healing is completed after 3 months, which indicates a previous heart damage had occurred prior to the latest stranding[26].

Previous studies have demonstrated the necessity of corroborating biochemical and histological findings with specific markers that might better delineate myocardial damage[10], so we compared the markers cTnI, cTnC, myoglobin and fibrinogen[10,11,15,44,55–59]. Immediately after acute ischemia caused by a vital trauma, muscle (skeletal and cardiac) proteins begin to leak as a result of early skeletal and myocardial cell membrane rupture, causing a rapid decline in myoglobin, cTnI, and cTnC contents, along with the deposition of plasma proteins, such as fibrinogen[10,11,15,55–57]. Therefore, damaged cardiomyocytes, in the absence of necrosis during myocardial injury, release cTnI and cTnC, resulting in an increase in serum levels and decreased cardiomyocyte troponin immunoreaction[15,40,60]. In the present study, the amount of damage present in cells was tested using immunohistochemical labelling of the animals that we were able to perform a complete necropsy and had not been previously frozen (animals 2, 4, 5, 6, and 9). All presented tissue depletion, especially cTnI and cTnC (Figs. 3–6) as well as

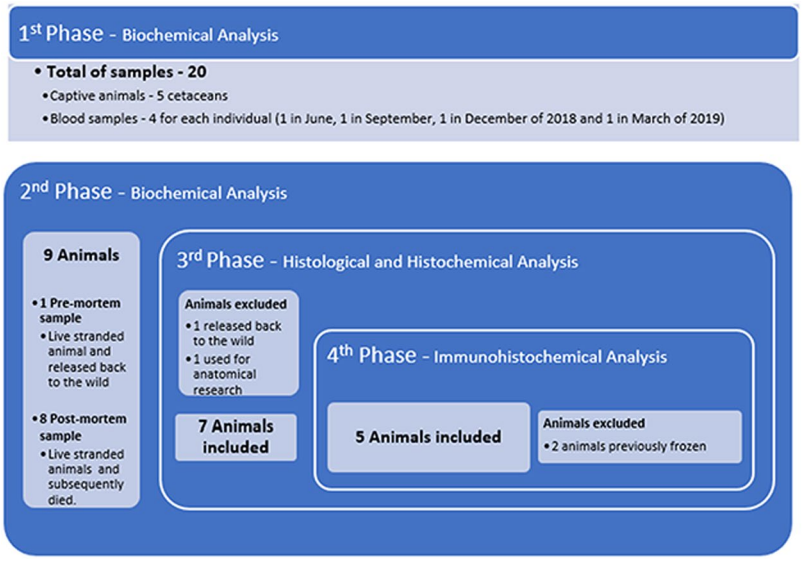

Figure 7. Diagram explaining the different stages of the present study.

myoglobin, together with intrafibrillar fibrinogen deposition. Consequently, with these immunohistochemical changes, we confirmed that the lesions present in these animals were ante mortem.

In conclusion, although the descriptions of CM has been reported in different species of cetaceans, characterisation of the cardiac damage in marine mammals – which is an important part of this syndrome – are still scarce. It is acknowledged and proven that live-stranding, itself, is an extreme and intensive stressor. Here, we describe the first clinical and pathological study of cardiac injury in live-stranded cetaceans – through biochemical analysis (especially of cTnI) and their histopathological, histochemical, and immunohistochemical correlations with decreased troponins immunolabeling – being the results highly comparable to the existing ones for SCMP in humans. We recommend further studies to advance our understanding and knowledge of the cardiac clinical-pathology of cetaceans.

Materials and Methods

Firstly, this report determines a baseline range for cardiac troponin I (cTnI), a specific cardiac biomarker for the detection of cardiac damage in cetaceans, more specifically bottlenose dolphins (*Tursiops truncatus*). Secondly, it describes the biochemical analysis and histological, histochemical, and immunohistochemical features of cardiac injuries associated with live-stranding in different species of cetaceans from the infraorder *Odontoceti*.

Phases and cases of the present study. The first phase of this study consisted in the determination of normal values for cTnI in cetaceans, specifically bottlenose dolphins, with a 95% probability through measurements obtained from captive animals (n = 5) at a local zoo. To achieve this, a total of 20 blood samples were collected, in June, September and December of 2018 and March of 2019 (4 samples of each individual, 1 sample in each month).

In the second phase, a total of 9 animals of four different species, including small and large odontocetes, were included in this study. Blood samples from these animals were collected, being one pre-mortem and the rest post-mortem since the animals subsequently died previous to or during the handling, restraint, transport, and/or rescue/rehabilitation (n = 8). All of the specimens were stranded alive on the coast of the Canary Islands from the beginning of 2016 until June of 2019.

During the third phase, a total of 7 animals were selected, from the animals included (n = 9) in the previous phase, for the histological and histochemical study, since we were not able to perform a necropsy in 2 animals, because one was released back to sea and another was used for anatomical research purposes.

In the fourth and last phase, 2 animals (from the prior phase) were eliminated from the study due to their prior freezing. Therefore, at the end of this phase, a total of 5 animals were studied with the use of the immunohistochemical technique for the detection of different markers.

In Fig. 7 we present the different stages formerly described. The stranding circumstance, basic and epidemiological data of each animal, included in this study, are detailed in Table 1 and Supplemental Table 2, respectively.

Biochemical analysis. Whole blood samples for each animal were collected in a gel tube (without anticoagulant), allowed to coagulate, and centrifuged at 3500 rpm for 5 min, two times to obtain the serum (approximately 1 ml).

Baseline range determination of cTnI in cetaceans. As the sample size is less than 40 individuals, we have had to assume that each of the variables is normally distributed and that for each individual, and for the variable (troponin), the measured value follows a model of the form $x_{ij} = \mu + b_i + \epsilon_{ij}$, where i is the index of the

individual (i from 1–5) and j is the order of measurement within the individual (j from 1–4)[61,62]. It has been assumed that the individual effect of the animal is $b_i \approx (0, \sigma_b)$, and that the variability within each animal is $\epsilon \approx N(0, \sigma_\epsilon)$, where the values of σ_b and σ_ϵ are specific to each variable. Under these assumptions, the values of each variable follow normal distributions of the form $X \approx (\mu, \sqrt{\sigma_b{}^2 + \sigma_\epsilon{}^2})$. The IMER library was used to estimate the parameter, and the anovaVCA library was used to estimate the variances, both using the statistical package R[63]. Once these quantities were estimated, the normality intervals were calculated according to the expression $[\mu - Z_{a/2}\sqrt{\sigma_b{}^2 + \sigma_\varepsilon{}^2}, \mu + Z_{a/2}\sqrt{\sigma_b{}^2 + \sigma_\varepsilon{}^2}]$, where $Z_{\alpha/2}$ is the $\alpha/2$ percentile of the normal distribution. Under all of these assumptions, the normal values of each of the variables considered can be expected to fall within this range with a probability of $1 - \alpha$. Therefore, we have constructed normal intervals with a 95% probability.

Biochemical analysis of the live-stranded cetaceans included in this study. On eight animals, blood samples were retrieved 1–38 h after the live-stranding and with a minimum of 2 h to a maximum of 24 h after the death of the animal. Additionally, one sample was recovered pre-mortem and within 5 h of the stressful event. The main biochemical markers analysed were cTnI, which reveals a specific heart injury, and CK, which demonstrates acute muscle damage, both cardiac and skeletal. The results of the biochemical analyses were compared to published values of different mammals, such as humans and domestic animals (dog), in respect to cTnI and diverse cetaceans of the infraorder *Odontoceti* in the case of CK whenever possible.

Histological and histochemical analysis. Complete necropsies were performed following the standard protocol, with the animals (n = 7) presenting a very fresh or fresh state of decomposition[2,64]. For histopathological analysis, the heart muscle (both atria and ventricles, different atrioventricular valves [bicuspid or mitral and tricuspid], and semilunar valves [sigmoid aortic and pulmonary] with the corresponding arteries) were fixed and processed. Specifically, 4 µm of the samples mentioned above were used for haematoxylin and eosin staining and Masson's trichrome techniques.

The sections subjected to the histochemical techniques were examined in a blind manner by three veterinarians (NC, ES, and PH). The sections were evaluated for vascular changes (congestion, haemorrhages, and interstitial oedema); acute degenerative changes (contraction band necrosis, wavy fibres, perinuclear vacuolisation, cytoplasmic hypereosinophilia, and pyknotic nuclei), as well as the presence of interstitial myoglobin globules, and infiltration of inflammatory cells. The extent of cardiac lesions was judged subjectively as follows: absent, mild, mild to moderate, moderate, moderate to severe, and severe.

Immunohistological analysis. Tissue sections of 3 µm, from each of the animals (n = 5), were immunolabeled using specific markers of myocardial injury, such as anti-cTnI, anti-cTnC, anti-myoglobin, and anti-fibrinogen primary antibodies, and were visualised using the VECTASTAIN® Elite ABC-Peroxidase Kit with reference PK-6100 (Vector Laboratories, Peterborough, United Kingdom). The methodology is summarised in Supplemental Table 3. For the immunohistochemical techniques, the positive control for cTnI and cTnC was a heart sample from a pig and a cetacean, respectively, with no apparent acute macroscopic and/or histological lesions, nor a live-stranding history in the case of the cetacean. For myoglobin and fibrinogen, we used a heart sample from a previously published case of a striped dolphin that was stranded alive and developed CM due to capture and the rehabilitation process[10,11]. Finally, the negative control was performed without the primary antibody.

The sections submitted to the immunohistochemical techniques were examined "blind" by three veterinarians (NC, ES, and PH). The amount of cell damage was confirmed as ante-mortem when it was accompanied with the immunohistochemically demonstrated depletion of myoglobin together with intrafibrillar fibrinogen deposition.

Evidence of ethical approval. Blood collection from the captive animals was carried out by specialised veterinarians authorised by "Palmitos Park" Zoo, within their routine prophylactic program, as well as their use for this scientific study, in compliance with the requirements established on the articles 3, 4 and 5 of the "Ley 31/2003, de 27 de octubre, de conservación de la fauna silvestre en los parques zoológicos" (BOE-A-2003-19800) and the Council Directive 1999/22/EC of 29 March 1999 relating to the keeping of wild animals in zoos (EUR-Lex -31999L0022). Likewise, the use of blood samples for this scientific study was expressly authorised by the Director of the "Palmitos Park" zoo.

Regarding the management of stranded cetaceans, required permission was issued by the environmental department of the Canary Islands' Government and the Spanish Ministry of Environment.

Data availability

All data reported in this work are classified and stored in the tissue bank of the Institute of Animal Health and Food Safety (IUSA). Veterinary School. University of Las Palmas de Gran Canaria.

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Author contributions

N.C. wrote the article, collected the blood samples, performed the necropsy of the animals and contributed towards the biochemical analysis and the gross, histological, histochemical and immunohistological description and diagnosis of the cases. E.S. and P.H. performed the necropsy of the animals and contributed towards the biochemical analysis and the gross, histological, histochemical, and immunohistological description and diagnosis of the cases and guided the N.C. during the drafting and publication process. A.F. performed the necropsy of the animals and contributed towards the gross and histological descriptions and diagnosis of the cases and guided N.C. during the publication process. M.A., M.A. and A.E.M. performed the necropsy of the animals and contributed towards the gross and histological descriptions and diagnosis of the cases. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-58497-3.

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Supplemental Tables of the manuscript:

Increased Plasma Cardiac Troponin I in Live-Stranded Cetaceans: Correlation with Pathological Findings of Acute Cardiac Injury

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Supplemental table 1. Summary of the cTnI values of the different blood samples collected from the 5 Bottlenose dolphins living in the Zoological Park.

	JUNE 2018	SEPTEMBER 2018	DECEMBER 2018	MARCH 2019
ANIMAL ZP 1	0.015	0.015	0,001	0.004
ANIMAL ZP 2	0.015	0.015	0.003	0.001
ANIMAL ZP 3	0.015	0.015	0.016	0.001
ANIMAL ZP 4	0.024	0.015	0.004	0.002
ANIMAL ZP 5	0.015	0.015	0.001	0.002

Supplemental table 2. Summary of the data of the cetaceans participating in the study.

	INTERNAL ANIMAL CODE	SAMPLES CODE (HISTOLOGICAL CODE)	SPECIES	SEX	AGE	PRESERVATION STATE	STRANDING LOCATION	TYPE OF STRANDING	PATHOLOGICAL ENTITY / CAUSE OF DEATH
ANIMAL 1 (*)	CET 810	(i907/16)	*Delphinus delphis*	Female	Juvenile	Fresh	Tenerife	Live	Pathology associated with significant loss of nutritional status
ANIMAL 2	CET 860	(i219/17)	*Delphinus delphis*	Female	Calf	Very Fresh	Lanzarote	Live	Neonatal/Perinatal pathology
ANIMAL 3 (†)	CET 893	(SA34/18)	*Stenella coeruleoalba*	Male	Adult	Fresh	Gran Canaria	Live	Undetermined
ANIMAL 4	CET 907	(SA90/18)	*Stenella coeruleoalba*	Male	Calf	Fresh	Gran Canaria	Dead	Pathology associated with significant loss of nutritional status
ANIMAL 5	CET 920	(SA220/18)	*Globicephala macrorhynchus*	Male	Juvenile	Fresh	Gran Canaria	Live	Pathology associated with significant loss of nutritional status
ANIMAL 6	CET 930	(SA256/18)	*Delphinus delphis*	Male	Adult	Fresh	Fuerteventura	Dead	Interaction with fishing activities
ANIMAL 7 (*)	CET 933	(SA336/18)	*Stenella coeruleoalba*	Male	Subadult	Fresh	Gran Canaria	Live	Pathology associated with significant loss of nutritional status
ANIMAL 8 (‡)	CET 935		*Stenella coeruleoalba*	Male	Juvenile	Alive	Gran Canaria	Live	Live animal
ANIMAL 9	CET 999	(SA279/19)	*Stenella coeruleoalba*	Female	Newborn	Very fresh	Gran Canaria	Live	Neonatal/Perinatal pathology

Detail of the data of the cetaceans participating in this study. (*) Frozen animals. (†) Animal used for anatomical research purposes. (‡) Animal released back into the sea.

Supplemental table 3. Summary of the immunohistochemical methodology used in this study.

ANTIGEN RETRIEVAL	SERUM	SOURCE	DILUTION	PRIMARY ANTIBODY	SOURCE	HOST	TYPE	DILUTION	SECONDARY ANTIBODY	SOURCE	DILUTION
Citrate buffer (*)	Swine serum (‡)	Dako (§)	10% (||)	Myoglobin (#)	Abcam (§§)	Rabbit	Polyclonal	1 in 200 (||||)	Polyclonal Swine Anti-Rabbit Immunoglobulins (‡‡‡)	Dako (§)	1 in 200 (§§§)
Citrate buffer (*)	Swine serum (‡)	Dako (§)	10% (||)	Fibrinogen (**)	Abcam (§§)	Rabbit	Polyclonal	1 in 50 (##)	Polyclonal Swine Anti-Rabbit Immunoglobulins (‡‡‡)	Dako (§)	1 in 200 (§§§)
Citrate buffer (†)	Swine serum (‡)	Dako (§)	10% (||)	Troponin I (††)	Abcam (§§)	Rabbit	Polyclonal	1 in 25 (***)	Polyclonal Swine Anti-Rabbit Immunoglobulins (‡‡‡)	Dako (§)	1 in 200 (§§§)
Citrate buffer (†)	Swine serum (‡)	Dako (§)	10% (||)	Troponin C (‡‡)	Abcam (§§)	Rabbit	Monoclonal	1 in 250 (†††)	Polyclonal Swine Anti-Rabbit Immunoglobulins (‡‡‡)	Dako (§)	1 in 100 (||||||)

Detail of the immunohistochemical protocol used in this study. (*) Citrate buffer, pH 6.0, 7 minutes at 96°C. (†) Citrate buffer, pH 6.0, 20 minutes at 100°C. (‡) Dako Swine serum (Normal) (X090110-8). (§) Dako (Glostrup, Denmark). (\|\|) Dilution of 10 µl of serum in 90 µl of PBS and incubated in a humidity chamber for half an hour. (#) Anti-Myoglobin antibody (ab187506). (**) Anti-Fibrinogen antibody (ab34269). (††) Anti-Cardiac Troponin I antibody (ab47003). (‡‡) Anti-Cardiac Troponin C antibody (ab137130). (§§) Abcam (Cambridge, United Kingdom). (\|\|\|\|) Dilution of 1 µl of antibody in 199 µl of serum at 1% in PBS and is incubated in a humidity chamber for at least 18 hours, inside the refrigerator. (##) Dilution of 1 µl of antibody in 49 µl of serum at 1% in PBS and incubated in a humidity chamber for at least 18 hours, inside the refrigerator. (***) Dilution of 1 µl of antibody in 24 µl of serum at 1% in PBS and incubated in a humidity chamber for at least 18 hours, inside the refrigerator. (†††) Dilution of 1 µl of antibody in 249 µl of serum at 1% in PBS and incubated in a humidity chamber for at least 18 hours, inside the refrigerator. (‡‡‡) Dako Polyclonal Swine Anti-Rabbit Immunoglobulins/Biotinylated (E035301-2). (§§§) Dilution of 1 µl of antibody in 199 µl of serum at 1% in PBS and incubated in a humidity chamber for half an hour. (\|\|\|\|\|\|) Dilution of 1 µl of antibody in 99 µl of serum at 1% in PBS and incubated in a humidity chamber for half an hour.

6.3 APPLICATION OF THE KNOWLEDGE OBTAINED IN THE DIAGNOSIS OF SPECIFIC CLINICAL CASES

The third specific objective of this study consisted in the presentation of two specific case studies of SCMP in cetaceans.

In this first case study we present a live-stranded specimen belonging to the *Mysticeti* infraorder, due to the fact that description of pathological entities and/or causes of death in the infraorder Mysticeti are still scarce. Therefore, we analysed a live-stranded neonatal Bryde's whale (*Balaenoptera edeni*) with fetal distress, which subsequently died soon after the stranding. This assessment was carried out through a physical exam, blood examination, gross necropsy evaluation, histopathology, and immunohistochemistry.

The results were published in the article (as followed) titled ***Skeletal and cardiac rhabdomyolysis in a live-stranded neonatal Bryde's whale with fetal distress***, on December 2019 in the *Frontiers in Veterinary Sciences* journal, Volume 20, Issue 6 (doi: 10.3389/fvets.2019.00476), having this journal an impact factor of 2.029 in 2018 and therefore being a Q1 in the veterinary category.

frontiers in Veterinary Science

CASE REPORT
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Skeletal and Cardiac Rhabdomyolysis in a Live-Stranded Neonatal Bryde's Whale With Fetal Distress

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The main objective of wildlife forensic investigation is to recognize pathologic changes and cause of death. Even though it may not always be possible to determine the specific illness and/or etiology, the description and subsequent interpretation of the injuries provide an invaluable understanding of pathology in cetacean post-mortem investigations. Although pathological studies have been previously reported in various cetacean species, such descriptions of the infraorder *Mysticeti* remain rare. A live-stranded neonatal Bryde's whale (*Balaenoptera edeni*) which subsequently died soon after the stranding, was assessed by physical exam, blood examination, gross necropsy evaluation, histopathology, and immunohistochemistry. It presented with elevated serum levels of creatine kinase, cardiac troponin I, urea, and creatinine. Microscopically, we observed keratin spicules (squamous epithelial cells) and areas of atelectasis in the lungs. Acute degeneration in the myocytes and cardiomyocytes were comparable to the findings previously described in cases of capture myopathy in live-stranded cetaceans. Immunohistochemistry biomarkers such as myoglobin, fibrinogen, and troponin were analyzed. Skeletal and myocardial damage has been documented in several cetacean species. However, this is the first reported case of skeletal and cardiac rhabdomyolysis associated with live-stranding in a newborn Bryde's whale that suffered from fetal distress.

Keywords: Bryde's whale (*Balaenoptera edeni*), cetaceans, rhabdomyolysis, live-stranding, *Mysticeti*, neonate, stress cardiomyopathy

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BACKGROUND

Pathological study of wildlife fauna has the disadvantage of an unknown clinical history of the animal. The complexity is enhanced in cetaceans because of the difficulty of performing clinical exams and/or other analyses on live animals. The most feasible technique for health assessment in dead cetaceans is by detection of injuries in these animals, through pathological study. The importance of these studies is recognized worldwide to promote conservation of these animals. Unfortunately, the description of pathological entities and/or causes of death in the infraorder *Mysticeti* remains rare (1–8).

Live-stranding is a pathological state with severe acute stress and physical damage central to its etiopathogenesis. It presents clinical and traumatic findings that can cause death of the animal, or can seriously aggravate an existing condition over the period of stranding, capture, handling, restraint, transportation and/or captivity (1, 2, 9–15). The response mechanisms and resultant damage to multiple systems involved in live-stranded cetaceans are comparable to exertional rhabdomyolysis (capture myopathy) in many animals, including birds and terrestrial or marine wild mammals (1, 9–11, 14, 16). Although pathological findings may vary among individuals, biochemical changes and histopathological lesions, consisting of ischemia-reperfusion injuries, are often observed. These changes result in local-to-generalized vasospasms and vasodilation (catecholamine surge, neurogenic shock, and impeded venous flow return by body compression), which is analogous to the stress cardiomyopathy in humans and in direct traumatic injury to muscles, resulting in acute to subacute degeneration (rhabdomyolysis). Acute renal failure associated with myoglobinuric nephrosis secondary to muscle damage and areas of necrosis in viscera are also observed (1, 9, 10, 12, 16–27).

In both wild and captive cetaceans, neonatal mortality is a recognized concern (28–31). The main causes of stranding and/or death in newborns are related with problems in pregnancy (abortion, prematurity), childbirth (fetal distress, dystocia), nursing (missed transfer of passive immunity), behavior (maternal-filial separation/maternal neglect), or intra and interspecific interactions with a fatal outcome. All these above are enclosed in the category of neonatal and/or perinatal pathologies (1, 2). In the case of asphyxia, the fetus responds with redistribution of the blood flow, which limits the deleterious effects of oxygen deprivation in vital organs. This enables the fetus to survive intact unless the asphyxia is profound or prolonged (32).

This report describes the biochemical analysis and gross, histopathological, histochemical, and immunohistochemical features in a live-stranded neonatal Bryde's whale.

CASE PRESENTATION

Stranding Circumstances

A 393-cm-long newborn male Bryde's whale was stranded on the coast of Fuerteventura, Canary Island, Spain, in September 2016. Observations between the high and low tide revealed that the animal appeared to be alive and few meters from the coast. At low tide, the animal was stranded on the beach and died before specialized assistance could be given.

Biochemical Analysis

A sample of whole blood was collected from the tail flukes, immediately post-mortem, for analysis of the serum. Biochemical markers of acute skeletal and heart muscle damage, creatine kinase (CK 460.0 U/L), and cardiac troponin I (0.20 μg/L), were analyzed. Kidney function was also assessed via blood urea nitrogen (BUN 162 mg/dL) and creatinine (2.4 mg/dL).

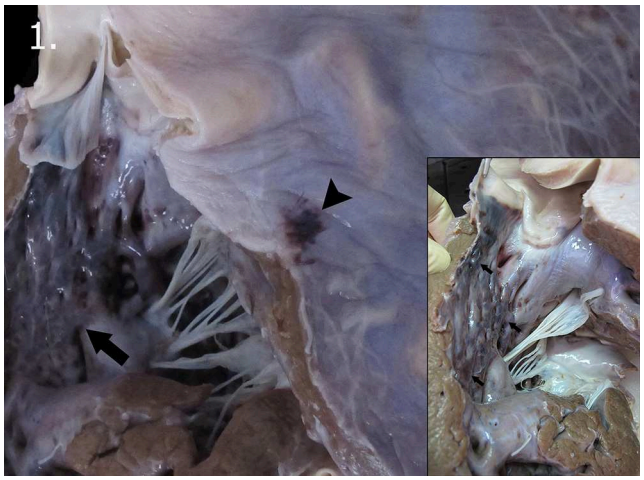

FIGURE 1 | Macroscopic vascular changes observed in the heart. Subepicardial (arrow head) and subendocardial hemorrhage (arrow) in the left ventricle. Detail of the subendocardial hemorrhages present in the left ventricle (arrows).

Gross Anatomic Analysis

A thorough necropsy was performed on the calf, following the standard protocol published by the European Society of Cetaceans and with the addition of some procedures detailed in the *Marine Mammals Ashore* manual, to determine the cause of death (14, 33). The animal was in poor body/nutritional condition with several linear erosions, distributed in a multifocal manner on the ventral abdomen (attributed to direct active stranding damage). While several vestigial hairs were noted in the lateral part of the maxilla, the navel was not healed and contained an internal white exudate. During dissection of the subcutaneous planes, moderate diffuse hemorrhages were observed, especially in the ventral region. The muscles were pale yellow-to-orange. The epiglottis was flaccid at the rostral level, the trachea showed mild-to-moderate foam, and the main and secondary bronchi and bronchioles presented abundant foam, representing pulmonary edema. Both lungs had multifocal and local extensive areas of dark reddish color and were firm (compatible with pulmonary atelectasis). Mild-to-moderate exudation of blood was noted at the incision. Some serous fluid was detected in the pericardial space. On sectioning, both ventricles presented moderate-to-severe subepicardial and subendocardial hemorrhage (**Figure 1**). The ductus arteriosus was also present. Moderate and diffuse mucosal congestion was observed in the stomach, liver, bladder, sclera, and meningeal and subarachnoid vessels. The cerebellum displayed moderate and diffuse congestion, edema, and mild-to-moderate hemorrhage.

Histopathological Analysis

Representative tissue samples were fixed in 10% formalin for ∼48 h and processed using standard protocol. The skeletal (*longissimus dorsi* and *rectus abdominis*) and heart muscles (both atria and ventricles), atrioventricular valves (bicuspid or mitral and tricuspid), semilunar valves (sigmoid, aortic, and

pulmonary with the corresponding arteries), and kidneys were examined for the potential presence of rhabdomyolysis and myoglobinuric nephrosis. Tissue sections (4-μm-thick) were used for hematoxylin and eosin and periodic Acid-Schiff staining, while 5-μm-thick layers were used for phosphotungstic acid, hematoxylin, and Masson's trichrome techniques.

On histopathologic and histochemical examinations, the skeletal and heart muscles presented with injuries consistent with vascular changes (i.e., hemorrhages and interstitial edema) and acute degenerative lesions (i.e., contraction band necrosis, wavy fibers, segmental hypercontraction, hypereosinophilia, cytoplasmic vacuolization, and nuclear pyknosis). Contraction band necrosis was observed in the *longissimus dorsi* and *rectus abdominis* (**Figure 2A**). Long and thin undulated fibers (wavy fibers) were noted (**Figure 2A**). Hypereosinophilia (**Figures 2B,C**) was usually associated with either segmental hypercontraction or segmental necrosis. The above-mentioned changes in the skeletal muscle were moderate and illustrated a multifocal pattern with smaller diameter fibers (presumably type I fibers). Atria and ventricles displayed a multifocal, moderate-to-severe degree of interstitial edema, wavy fibers, hypereosinophilia, and cytoplasmic vacuolization with pyknotic nucleus (**Figure 2D**). Both ventricles demonstrated mild-to-moderate, multifocal, subepicardial, and subendocardial hemorrhage (**Figure 2E**).

Additional histopathological findings include (a) discrete capsular hemorrhage and mild-to-moderate, diffuse congestion in the kidneys with (b) mild, multifocal dilatation of the renal tubules; (c) in the lungs, severe, multifocal presence of keratin spicules (squamous epithelial cells) in alveolar spaces (**Figure 2F**) and (d) severe, multifocal, local extensive areas of atelectasis and moderate, multifocal alveolar hemorrhages; (e) severe, diffuse macro and microvacuolar degeneration (hyaline globules) in the hepatocytes; (f) moderate, focal suppurative omphalitis with the presence of coccoid bacterial colonies in the most superficial areas of the navel, (g) mild congestion and hemorrhage in multiple organs.

Immunohistological Analysis

Tissue sections (3 μm thick) were immunolabeled with anti-myoglobin (skeletal and cardiac muscles, and the kidneys), anti-fibrinogen (skeletal and cardiac muscles), anti-cardiac troponin I (cardiac muscle), and anti-cardiac troponin C (cardiac muscle) primary antibodies. They were visualized using the VECTASTAIN® Elite ABC-Peroxidase Kit (PK-6100) from Vector Laboratories (Peterborough, United Kingdom). The immunohistochemical methodology is summarized in **Supplementary Table 1**. The negative control for the latter consisted of serial sections of the heart without the primary antibody. In contrast, the positive control for myoglobin and fibrinogen were from a cetacean heart sample of a striped dolphin (*Stenella coeruleoalba*). The dolphin had been stranded alive and developed CM owing to capture and human interaction during the rehabilitation process (9, 10). Heart samples from a pig and cetacean, with no apparent acute macroscopic and/or histological lesions, were used as positive controls for cardiac troponin I and cardiac troponin C.

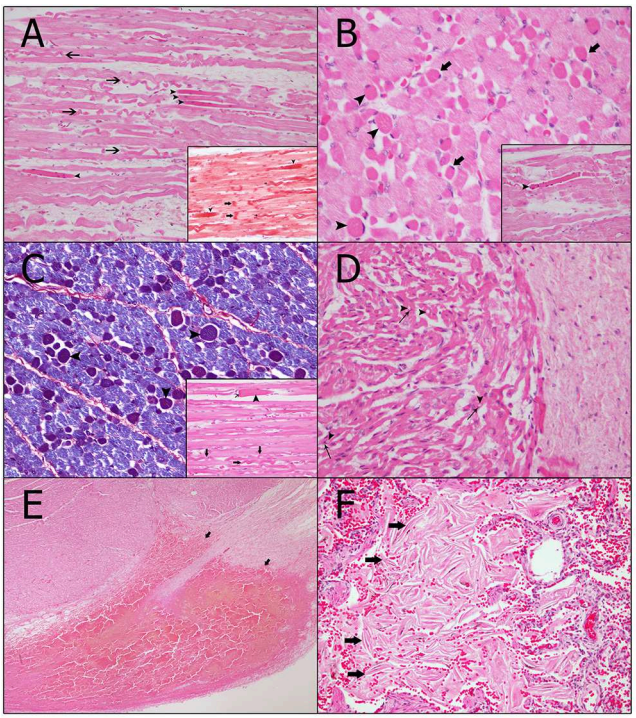

FIGURE 2 | Vascular and acute degenerative changes observed in the skeletal and heart muscles. **(A)** Long and thin undulated fibers, also referred to as wavy fibers (arrows), can be seen in the myocytes. In addition, the myocytes demonstrate hypereosinophilia, i.e., an increase in staining of necrotic muscular cells (arrow heads) with different histochemical techniques, which is usually associated with segmental hypercontraction (arrow heads) (hematoxylin and eosin technique, magnification: 40×). Inset: The contraction band necrosis (arrows) runs transversely throughout the myocytes and is identified via the increasing red color intensity (Masson's trichrome technique). Furthermore, both hypereosinophilia and wavy fibers can be observed (thin arrows) (magnification: 40×). **(B)** In the transversal cut, myocytes of minor caliber (most likely type I fibers) are affected and present hypereosinophilia with segmental hypercontraction (arrow heads). Moreover, endomysia edema (thin arrows) can be seen (hematoxylin and eosin technique, magnification: 60×). Inset: Detail of the segmental hypercontraction of a myocyte (hematoxylin and eosin technique, magnification: 40×). **(C)** More intense blue coloring of the damaged myocytes of minor caliber, which identifies hypereosinophilia and segmental hypercontraction (arrow heads) (phosphotungstic acid hematoxylin technique, magnification: 40×). Inset: Detail of a myocyte with segmental hypercontraction (arrow head) and segmental necrosis of the fiber with the retraction cap (thin arrow). Wavy fibers can also be observed (arrows) (hematoxylin and eosin technique, magnification: 40×). **(D)** Cardiomyocytes show vacuolar degeneration (arrow heads) and pyknotic nucleus (thin arrows) (hematoxylin and eosin technique, magnification: 40×). **(E)** Vascular changes present in the heart consistent with a subepicardial hemorrhage in the left ventricle (arrows) (hematoxylin and eosin technique, magnification: 4×). **(F)** Detail of the intra-alveolar keratin spicules (arrows) (hematoxylin and eosin technique, magnification: 40×).

Immunohistochemically, the degenerated/necrotic muscular and heart cells showed homogenous, intrafibrillar depletion of cardiac troponin I, cardiac troponin C (**Figure 3A**), and myoglobin. Damaged cells from the skeletal and cardiac muscles were found to exhibit several concentrations of immunolabeling for fibrinogen (**Figure 3B**). The kidneys did not exhibit any accumulation of myoglobin.

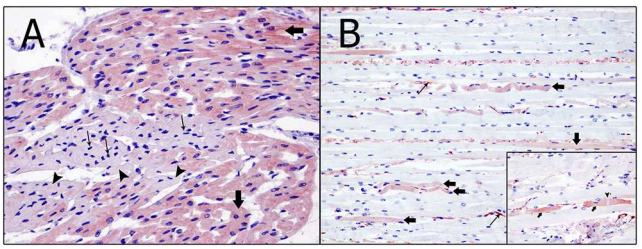

FIGURE 3 | Immunohistochemical techniques in skeletal and heart muscles. **(A)** Degenerated/necrotic cardiomyocytes (arrow heads), with pyknotic nucleus (thin arrows), show intrafibrillar depletion of cardiac troponin C. In contrast, normal cardiomyocytes (arrows) present an intense immunolabeling (immunohistochemical technique: anti-troponin C, magnification: 60×). **(B)** Expression of fibrinogen (arrows) in the myocytes presenting changes, including wavy fibers, hypereosinophilia and segmental hypercontraction. Immunolabeling of fibrinogen in the interior of the blood vessels can also be seen (thin arrows) (immunohistochemical technique: anti-fibrinogen, magnification: 40×). Inset: Necrotic myocytes (arrows) strongly expressed alongside the contraction band necrosis (arrow head) and inside the blood vessels (thin arrows) (immunohistochemical technique: anti-fibrinogen, magnification: 40×).

DISCUSSION

Discussion of the Animal Characteristics

Cetacean newborns/neonates are defined as having a compatible total length, displaying "fetal folds" over the body, soft and folded dorsal fin and tail flukes, vibrissal hairs or vibrissal crypts, and a healing (or closing) navel (34). Our animal was 393 cm long, slightly under the normal range (395–430 cm) (35). The animal did not present any "fetal folds" but we identified some vibrissal hairs; the navel was not healed and contained inflammatory exudate. This suggested an infection after birth; therefore, the animal would most likely be a few days old (34).

Discussion of the Biochemical Results

Clinico-pathological evaluation was challenging in this case as biochemical values, such as that for cardiac troponin I, are rarely reported. They may not exist in the scientific database for various species of cetaceans, particularly the infraorder *Mysticeti*. Our biochemical data was compared with published papers assessing different mammals, including humans, dogs, and other species of cetaceans [e.g., bottlenose dolphins (*Tursiops truncatus*) belonging to the infraorder *Odontoceti* and a stranded baby gray whale (*Eschrichtius robustus*) of the infraorder *Mysticeti*]; this is summarized in **Table 1** (13, 36–39).

In concordance with the results obtained from other species (i.e., 107–255 U/L in a neonatal gray whale), injury to the skeletal muscle was supported by an increase in CK (460 U/L), which is one of the useful indicators of both skeletal and cardiac muscle damage (12, 13, 16, 39–41). After myocardial injury, CK begins to rise in 4 to 9 h, peaks at 24 h, and returns to baseline 48 to 72 h after the stress event (42). This increase in CK was correlated with histological and immunohistochemical changes, thus supporting the presence of muscle damage.

Cardiac troponin I, measured by conventional assays, is elevated in >90% of patients with stress cardiomyopathy (43).

TABLE 1 | Comparison between the biochemical results of the studied animal and normal laboratory values of other mammals.

	Class *Mammalia*							
	Order *Primates*	**Order *Carnivora***		**Order *Cetartiodactyla***				
	Suborder *Haplorhini*			**Suborder *Cetacea***				
	Infraorder *Simiformes*			**Infraorder *Odontoceti***		**Infraorder *Mysticeti***		
	Family *Hominidae*	**Family *Canidae***		**Family *Delphinidae***		**Family *Eschrichtiidae***	**Family *Balaenopteridae***	
	HUMANS (*Homo sapiens*) (17)	**DOG (*Canis lupus familiaris*) (19, 20)**		**BOTTLENOSE DOLPHIN (*Tursiops truncatus*) (9)**		**NEONATAL GRAY WHALE (*Eschrichtius robustus*) (9)**	**NEONATAL BRYDE'S WHALE (*Balaenoptera edeni*)**	
				Captive (*n* = 38)	**Wild Atlantic Juvenile (*n* = 96)**	**Stranded (*n* = 1)**	Stranded (n = 1)	
Creatine Kinase (U/L)	30–170	0–190		100–250	47–455	107–255		**460**
Troponin I (μg/L)	≤0.1	≤0.03–0.07		ND	ND	ND		**0.20**
Blood Urea Nitrogen (mg/dL)	8–20	7–20.72		42–58	42–77	21–75		**162**
Creatinine (mg/dL)	0.7–1.3	0.44–1.595		1.0–2.0	0.68–1.49	1.0–2.0		**2.4**

Our animal presented with 0.20 μg/L cardiac troponin I in the serum; this is higher than the reference values in both humans (≤0.1 μg/L) and dogs (≤0.03–0.07 μg/L) (36–38). The release of troponin from injured cardiomyocytes usually occurs 3 to 9 h after ischemic damage, peaks in 12 to 48 h, and remains elevated for 4 to 7 days (42, 44, 45). Hence, the above laboratory values cannot aid in early detection of myocardial necrosis (1–3 h). These markers do not assist in accurate diagnosis until 6 or more hours after the onset of the event. In order to obtain a satisfactory clinical picture in humans, blood should be drawn 6–9 h after the onset of the stress event and/or symptom onset (44). The increase in cardiac troponin I serum levels and the decrease in myocyte troponin immunoreaction is caused by the early release of cardiac troponin I and troponin C by damaged cardiomyocytes. This was verified by immunohistochemistry (22, 46).

In this case, pre-renal azotemia is likely related to hypovolemic shock, and was reasonably supported by higher BUN values (162 mg/dL) than the reference values from the other species (i.e., 21 to 75 mg/dL in the stranded gray whale). Hypovolemic shock can be originated by a relative decrease in the effective circulating volume without a loss of total body fluid (i.e., decreased in venous return) and/or a direct intravascular fluid loss (i.e., dehydration or hemorrhage) (12, 40). In our case, we can associate this clinical finding with various causes, such as compartment syndrome, heart failure, and dehydration. Moreover, creatinine was slightly above (2.4 mg/dL) the normal values seen in other species (i.e., 1.0 to 2.0 mg/dL in a stranded neonatal gray whale). In order to confirm the hypothesis that the increase in creatinine we observed could be within the normal ranges for the Bryde's whale species, more accurate age and species-specific normal values would be needed. Small elevation of creatinine and high levels of urea can be associated with pre-renal azotemia caused by dehydration. This is a possible interpretation for our case. Since dehydration is associated with prolonged fasting, it is important to consider this aspect in stranded animals, especially young ones (47).

Discussion of the Histopathological Results

With the anatomopathological findings, the morphological diagnoses include (1) severe multifocal fetal atelectasis with presence of severe multifocal keratin spicules and a moderate alveolar hemorrhage; (2) multifocal moderate-to-severe acute degeneration of cardiomyocytes; (3) moderate multifocal acute skeletal muscle degeneration; and (4) mild congestion and hemorrhage in various organs.

Atelectasis is a relatively frequent finding in fetal and neonatal (*atelectasis neonatorum*) deaths (perhaps associated with aspiration of amniotic fluid or meconium), it is found incidentally or in non-specific forms in young or adult individuals (1, 48–50). In contrast, pulmonary edema with intra-alveolar keratin spicules (*pulmonary vernix caseosa*), either isolated or in aggregates of stratified epithelium, keratinized and with nuclear retention, can be observed in fetal distress. Considering non-specific findings, without knowledge of the primary cause, and after discarding lesions compatible with other etiological diagnoses, we considered the macro and microscopic findings as a whole to be typical of fetal distress (1).

Acute degenerative changes, such as contraction band necrosis, were observed in both the *longissimus dorsi* and the *rectus abdominis*. This represents a skeletal and myocardial lesion characteristic of transient ischemia and reperfusion, which is associated with high concentrations of endogenous catecholamines (23, 51, 52). This condition has been reported in humans after stressful events, as well as in other animals after acute death, including seals and cetaceans (1, 9–12, 16–23, 53–56). Wavy fibers were also detected in the skeletal and heart muscles. Considering that they are the first histologic abnormality associated with ischemia, this condition may be used as a morphological indicator of early myocardial injury (9–11, 22, 23, 53, 57). In addition, hypereosinophilia was observed in the skeletal and cardiac muscles, respectively. Animals that die following a stressful situation present with this cytoplasmic alteration (9–11, 22, 53, 57, 58). The animal also presented with vacuolization in the cardiomyocytes. Previous studies commonly associate this with areas that experience severe, chronic, and fatal ischemia as a result of acute death due to stressful situations (11, 22, 53, 59).

Vascular changes, including congestion, interstitial edema, and hemorrhage, are generally detected through histological approaches and form part of the stress cardiomyopathy pathology (17). In the current case, all the heart sections showed separated fibers, with interstitial edema. Following the introduction of catecholamines, interstitial edema is usually associated with subendocardial and subepicardial hemorrhage, found in both ventricles of this neonate (57). These lesions are occasionally detected in humans with stress cardiomyopathy and have been previously demonstrated in animals that died after live-stranding and handling (1, 9, 10, 17, 20).

The sequence of changes in an acute ischemic injury begins within 5 min. The myocardium reveals long, thinned, wavy fibers separated by spaces, characterizing edema and microvascular congestion at the borders of the ischemic myocardium. In 2 to 3 h, early changes of cardiomyocyte coagulation necrosis with nuclear pyknosis, color change, more specifically "brick red change" or cytoplasm hypereosinophilia, focal contraction bands, and subtle interstitial edema are evident. Hypereosinophilia and edema become more pronounced and more easily recognizable 3 to 6 h after the event. Six to 12 h later changes accelerate and more extensive contraction band necrosis with reperfusion is noted (25). Based on the acute degenerative findings in both skeletal and cardiac muscle of our case, we propose that the ischemic injury, which caused these lesions, occurred between 6 and 12 h prior to death, coinciding with the live-stranding.

Discussion of the Immunohistochemical Results

Previous studies demonstrated the necessity of corroborating histopathological findings with specific markers to better determine the amount of damage present in cells. The immunohistochemical confirmation ante-mortem showed depletion of myoglobin, cardiac troponin I, and cardiac troponin C as well as intrafibrillar fibrinogen deposition (9, 10, 12, 22, 45, 58, 60–63). Depletion of myoglobin (a

marker used for skeletal and cardiac damage), troponin I and C (specific markers to detect injury to the heart), as well as accumulation of fibrinogen (used to identify skeletal and cardiac damage) in injured cells was confirmed in the present study.

Although this animal presented with clinical and pathological findings resembling rhabdomyolysis, which can lead to a secondary myoglobinuric nephrosis, lesions associated with acute kidney injury (i.e., intrinsic kidney disease/ damage or acute tubular necrosis) were not detected through the histopathological and immunohistochemical studies (1, 9, 10, 12, 16–27, 64).

Discussion of the Cause of Death

Considering the biochemical results and the macro and microscopic findings which concur with the etiological diagnoses of fetal distress and skeletal and cardiac rhabdomyolysis, we propose that the most probable cause of death in this animal is active stranding pathology which aggravated a previous neonatal/perinatal pathology.

The active stranding pathology is defined by a set of lesions and biochemical findings in animals that were stranded alive and leads to both a catecholaminergic crisis (stress cardiomyopathy) and multi-organ ischemic-reperfusion damage with rhabdomyolysis with myoglobinuric nephrosis secondary to muscle damage. The severity of this syndrome usually causes the death of the animal, occasionally as a result of the intensification of preexisting pathologies (1, 9, 10, 22).

Neonatal/perinatal pathology in cetaceans comprises a wide constellation of etiologic factors, including fetal distress (1, 2). A severe disturbance in the oxygen supply to the fetus can have effects on the newborn's cardiac function. Elevated levels of cardiac troponin I, cardiac troponin T, CK and its fraction MB can be observed in full-term infants after intrauterine hypoxia and respiratory distress (65). Limited studies have shown premature infants and various breeds of stillborn cow calves to present with acute degenerative changes, such as myocardial necrosis, which may result from antepartum or intrapartum asphyxia (hypoxia) (65–67).

CONCLUSIONS

Although we cannot confirm that the elevated serum values (CK, cTnI, BUN, and creatinine) were due to post stranding or fetal distress, but based on the histological findings, we can conclude that these lesions are due to live stranding. Therefore, we suggest that the animal assessed here probably died because of an exacerbation of preceding injuries (fetal distress) and the final complications of stranding. Since description of pathological entities and/or causes of death in the *Mysticeti* infraorder is still scarce, we consider this article to be an important contribution to improve conservation efforts by reducing the mortality of these animals.

DATA AVAILABILITY STATEMENT

All data reported in this work is classified and stored in the tissue bank of the Institute of Animal Health and Food Safety (IUSA), Veterinary School, University of Las Palmas de Gran Canaria (ULPGC).

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because no experiments were performed on live animals. Required permission for the management of stranded cetaceans was issued by the environmental department of the Canary Islands' Government and the Spanish Ministry of Environment. No experiments were performed on live animals.

AUTHOR CONTRIBUTIONS

NC wrote the article, collected the blood sample, performed the necropsy of the animal, and contributed toward the biochemical analysis, gross, histological, histochemical, and immunohistological description and diagnosis of the case. ES and PH contributed toward the biochemical analysis, gross, histological, histochemical, and immunohistological description and diagnosis of the case, and guided NC during the drafting and publication processes. AF, CS-S, RP-L, and MA performed the necropsy of the animal and contributed toward the gross and histological description and diagnosis of the case. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2019.00476/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Table 1. Summary of the immunohistochemical methodology used in this study.

ANTIGEN RETRIEVAL	SERUM	SOURCE	DILUTION	PRIMARY ANTIBODY	SOURCE	HOST	TYPE	DILUTION	SECONDARY ANTIBODY	SOURCE	DILUTION
Citrate buffer (1)	Swine serum (3)	Dako (4)	10% (5)	Myoglobin (6)	Abcam (10)	Rabbit	Polyclonal	1 in 200 (11)	Polyclonal Swine Anti-Rabbit Immunoglobulins (15)	Dako (4)	1 in 200 (16)
Citrate buffer (1)	Swine serum (3)	Dako (4)	10% (5)	Fibrinogen (7)	Abcam (10)	Rabbit	Polyclonal	1 in 50 (12)	Polyclonal Swine Anti-Rabbit Immunoglobulins (15)	Dako (4)	1 in 200 (16)
Citrate buffer (2)	Swine serum (3)	Dako (4)	10% (5)	Troponin I (8)	Abcam (10)	Rabbit	Polyclonal	1 in 25 (13)	Polyclonal Swine Anti-Rabbit Immunoglobulins (15)	Dako (4)	1 in 200 (16)
Citrate buffer (2)	Swine serum (3)	Dako (4)	10% (5)	Troponin C (9)	Abcam (10)	Rabbit	Monoclonal	1 in 250 (14)	Polyclonal Swine Anti-Rabbit Immunoglobulins (15)	Dako (4)	1 in 100 (17)

Detail of the immunohistochemical protocol used in this study. (1) Citrate buffer, pH 6.0, 7 minutes at 96ºC. (2) Citrate buffer, pH 6.0, 20 minutes at 100ºC. (3) Dako Swine serum (Normal) (X090110-8). (4) Dako (Glostrup, Denmark). (5) Dilution of 10 µl of serum in 90 µl of PBS and incubated in a humidity chamber for half an hour. (6) Anti-Myoglobin antibody (ab187506). (7) Anti-Fibrinogen antibody (ab34269). (8) Anti-Cardiac Troponin I antibody (ab47003). (9) Anti-Cardiac Troponin C antibody (ab137130). (10) Abcam (Cambridge, United Kingdom). (11) Dilution of 1 µl of antibody in 199 µl of serum at 1% in PBS and is incubated in a humidity chamber for at least 18 hours, inside the refrigerator. (12) Dilution of 1 µl of antibody in 49 µl of serum at 1% in PBS and incubated in a humidity chamber for at least 18 hours, inside the refrigerator. (13) Dilution of 1 µl of antibody in 24 µl of serum at 1% in PBS and incubated in a humidity chamber for at least 18 hours, inside the refrigerator. (14) Dilution of 1 µl of antibody in 249 µl of serum at 1% in PBS and incubated in a humidity chamber for at least 18 hours, inside the refrigerator. (15) Dako Polyclonal Swine Anti-Rabbit Immunoglobulins/Biotinylated (E035301-2). (16) Dilution of 1 µl of antibody in 199 µl of serum at 1% in PBS and incubated in a humidity chamber for half an hour. (17) Dilution of 1 µl of antibody in 99 µl of serum at 1% in PBS and incubated in a humidity chamber for half an hour.

6.4 APPLICATION OF THE KNOWLEDGE OBTAINED IN THE DIAGNOSIS OF SPECIFIC CLINICAL CASES

As mentioned in the previous point the third specific objective of this study consisted in the presentation of two specific case studies of SCMP in cetaceans.

In this second case study we studied the biochemical analysis of consecutive blood samples in vivo, followed by the gross, histopathological, histochemical and immunohistochemical features caused by the live-stranding and consequent rehabilitation attempt, for a certain period of time, in a juvenile male Risso's dolphin (*Grampus griseus*).

The results were published in the article (as followed) titled ***Capture myopathy and stress cardiomyopathy in a live-stranded Risso's dolphin (Grampus griseus) in rehabilitation***, on January 2020 in the *Animals* journal*,* Volume 10, Issue 2 (doi: 10.3390/ani10020220), having this journal an impact factor of 1.832 in 2018 and therefore being a Q1 in the veterinary category.

animals

MDPI

Case Report

Capture Myopathy and Stress Cardiomyopathy in a Live-Stranded Risso's Dolphin (*Grampus griseus*) in Rehabilitation

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Simple Summary: Free-living cetaceans are threatened, daily, by a wide variety of stressful situations. An example is provided by live-stranding, in which a cetacean is alive on the beach or in shallow water, and unable to free itself and resume its normal activity. This is the first case of capture myopathy and stress cardiomyopathy in a live-stranded juvenile male Risso's dolphin (*Grampus griseus*) with subsequent rehabilitation attempted. Valuable use of blood samples, and finally necropsy assessments, advances our understanding about the pathology common in live-stranded cetaceans.

Abstract: Capture myopathy (CM) is described in wild animals as a metabolic syndrome resulting from the extreme stress suffered during and after capture, handling, restraint, and transport. Although CM has been characterized in many species of cetaceans, descriptions of cardiac injury—an important component of this syndrome, and, according to previous authors, comparable to the existing human pathology so-called stress cardiomyopathy (SCMP)—are still rare. Therefore, the main aim of this report is to illustrate, for the first time, the biochemical analysis, and gross, histopathological, histochemical and immunohistochemical features of CM, and more specifically of the SCMP involved in this syndrome, caused by the live-stranding and consequent rehabilitation attempt, for a certain period of time, in a juvenile male Risso's dolphin (*Grampus griseus*). The animal presented elevated values of creatine kinase, cardiac troponin I and blood urea nitrogen, with some variations during the rehabilitation period. Histologically, we detected vascular changes and acute degenerative lesions analogous to the ones observed in humans with SCMP. We consider this study to be an important contribution to the study of cetaceans since it could help in decision-making and treatment procedures during live-strandings and improve conservation efforts by reducing the mortality of these animals.

Keywords: animal conservation; animal welfare; cetaceans; biochemistry; histopathology; immunohistochemistry

1. Introduction

Stress is present in daily life, and all life forms have evolved mechanisms to cope with stressful situations [1]. It is commonly defined as the adaptive changes (or stress responses) caused when a person or animal perceives a threat to their homeostasis [2,3].

In human pathology, despite still under-recognized and often misdiagnosed, an unique reversible cardiac syndrome, triggered by a stressful event, has globally been reported and described as stress cardiomyopathy (SCMP) [4,5].

The clinical presentation is similar to the acute myocardial infarction. Diagnostic criteria for SCMP has been established by the Heart Failure Association (electrocardiography, echocardiography, cardiac catheterizations, and biochemical analysis). Regarding the biomarkers, cardiac troponin I (cTnI) and/or creatine kinase (CK) have small, rapid increases to above normal levels [4–7].

Histological findings are consistent with ischemia-reperfusion injury, such as vascular changes (i.e., congestion, hemorrhages, interstitial edema) and acute necrotic degenerative lesions (i.e., contraction band necrosis, wavy fibers, hypereosinophilia and cytoplasmic vacuolization) [4,5,8].

Cetaceans are exposed daily to a wide variety of stressful situations (i.e., live-stranding, ship collisions, bycatch) that influence their well-being. Efforts have been made to reduce the impact of these situations, but whales and dolphins continue to be threatened [9–15]. Live-stranding is an unnatural and distressful situation, where the cetacean is alive on the beach or in shallow water, being unable to resume normal activity [16–18]. Independently of the animal's previous health, this implies an anomalous and extreme situation for an organism that is not adapted to terrestrial environmental conditions. Therefore, it is considered life-threatening as it may cause death or seriously aggravate a previous disease [9–12,14,16,18,19].

The acute and stressful deaths of live-stranded cetaceans might be attributed to the "stress response syndrome" or "alarm reaction" which are thought to be comparable to those described in capture myopathy (CM), in which the cardiac damage, due to the extreme stress, seems to have an important role. According to some authors, cetaceans are especially predisposed to develop SCMP, similarly to the SCMP in humans. It is the most devastating form of acute stress described in several animals that may occur during and after the capture, handling, restraint, and transport [9,10,14]. Although the pathological findings vary amongst individuals, the biochemical changes are consistent with elevated serum muscle enzyme activities, specifically CK, and elevated blood urea nitrogen (BUN). There are no biochemical studies on *Odontoceti* in which specific cardiac markers have been analyzed. Histopathological changes consisting of ischemia-reperfusion injuries may be observed. These changes result in local-to-generalized vasospasms and vasodilation (as seen in human SCMP), as well as in direct traumatic injury to muscles (rhabdomyolysis), with acute renal failure associated with myoglobinuric nephrosis secondary to muscle damage [9–12,15,20,21].

The aim of the present report is to illustrate, for the first time, the biochemical analysis, gross, histopathological, histochemical and immunohistochemical features of the CM, and more specifically of the SCMP involved in CM, caused by the live-stranding and consequent rehabilitation attempt in a juvenile male Risso's dolphin (*Grampus griseus*).

2. Materials and Methods

This study is of a juvenile male Risso's dolphin (*Grampus griseus*) which was stranded alive on the coast of Gran Canaria (Canary Islands, Spain), on April 26, 2019 (notification at 8:30 a.m.). After receiving specialized first aid (i.e., help maintaining correct body posture and wetness of skin, evaluating breathing and heart rate) at the location, the animal was transferred to the Wildlife Recovery Center (Gran Canaria), where it was monitored 24 h a day. From day 0 (26 April 2019) to day 5 (1 May 12019), several diagnostic tests (i.e., biochemical and hematological analysis, gastric probing and ultrasound evaluation) were performed to establish the most appropriate treatment. Around 10 pm on 29 April 2019 (day 3), the animal began to have muscle spasms. The next day, a lateral body curvature began to develop at the caudal peduncle, with increasing muscle tremors. Due to the worsening prognosis the animal was euthanized on 1 May 2019 (day 5). The therapeutic treatment and the euthanasia protocol applied in this animal are described in the Supplementary Materials Table S1 and Table S2, respectively.

2.3. Evidence of Ethical Approval

Permission for the management of stranded cetaceans was issued by the environmental department of the Canary Islands' Government and the Spanish Ministry of Environment.

2.2. Biochemical Analysis

Samples of whole blood were collected from the tail flukes, into a gel tube (without anticoagulant), were allowed to coagulate, and then centrifuged twice, each time at 3500 rpm for 5 min to obtain the serum for analysis. The first blood sample was collected 4 h after the warning (approximately midday), the remaining samples every 24 h during rehabilitation, and also post-mortem (1:30 h after euthanasia). The biomarkers for acute skeletal and heart muscle damage (CK and cTnI) and the kidney function (BUN and creatinine) were analyzed.

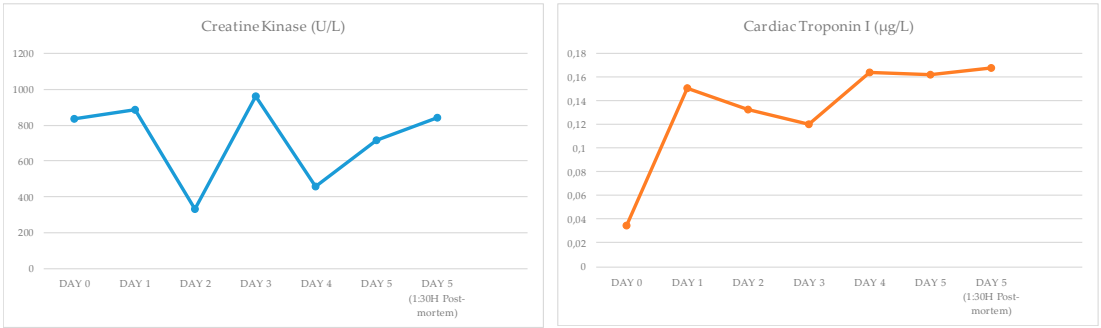
2.3. Gross, Histological, Histochemical and Immuznohistochemical Analysis

Prior to the necropsy, and after the euthanasia, an entire body scan was carried out, using helical computer tomography (CT) (Toshiba Astellion 16) setting the protocol on 120kV, 300 mA and a reconstruction slice thickness/slice interval of 1/0,80 mm. All the DICOM files obtained were processed with the Horos software. Thereafter, a standard protocol full necropsy was performed, where representative tissue samples were collected and fixed in 10% formalin for approximately 48 hours and processed using standard protocol [16,22]. Due to the potential presence of CM and SCMP, the skeletal (*longissimus dorsi* and *rectus abdominis*) and heart muscles (both atria and ventricles), atrioventricular valves (bicuspid or mitral and tricuspid), semilunar valves (sigmoid aortic and sigmoid pulmonary with the corresponding arteries), and kidneys were fully studied. Tissue sections (4- and 5-µm-thick) were used for hematoxylin and eosin (HE) and Masson's trichrome technique, respectively, while 3-µm-thick layers were immunolabeled with anti-myoglobin, anti-fibrinogen, anti-cardiac troponin I, and anti-cardiac troponin C primary antibodies. The followed protocol was presented in a previous publication, and it can be accessed in Supplementary Materials Table S3 [15].

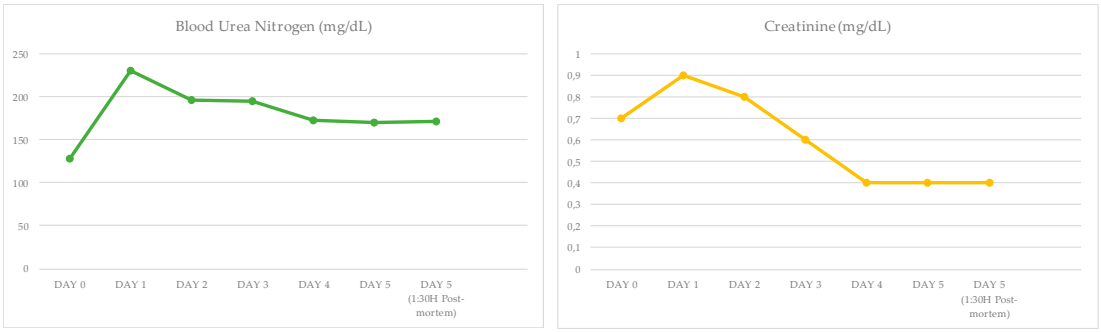
3. Results

3.1. Biochemical Results

The different measurements obtained during the rehabilitation time and after euthanasia, are presented in Figure 1 and in the Supplementary Materials Table S4.



(**a**) (**b**)



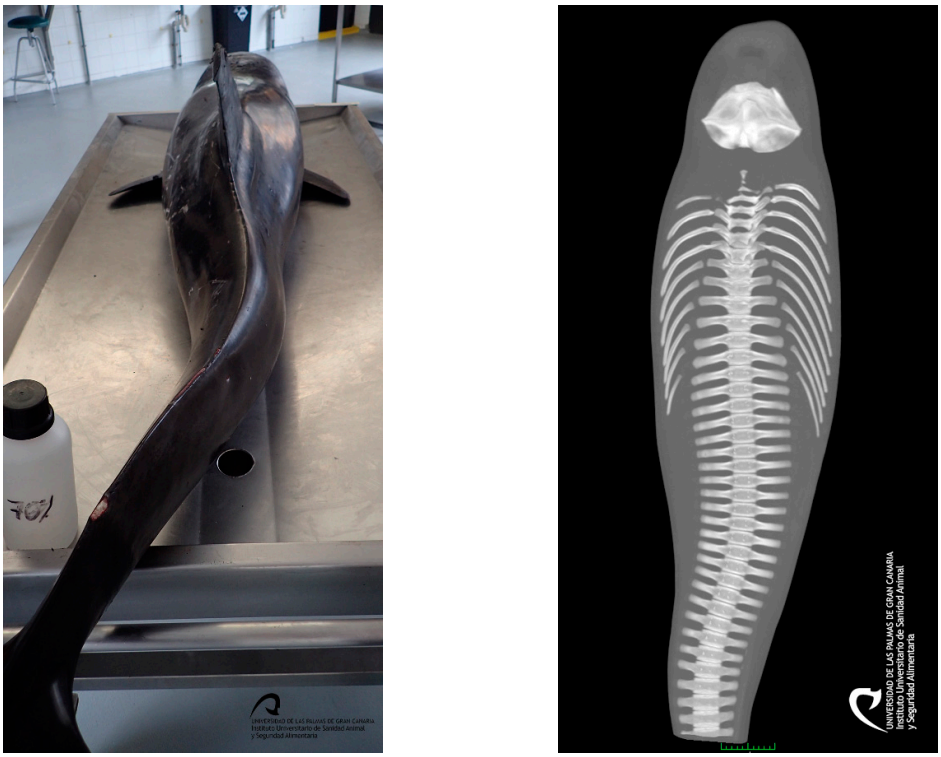
(**c**) (**d**)

Figure 1. Biochemical analysis during rehabilitation and after euthanasia: (**a**) Creatine kinase (CK); (**b**) Cardiac troponin I (cTnI); (**c**) Blood urea nitrogen (BUN); (**d**) Creatinine.

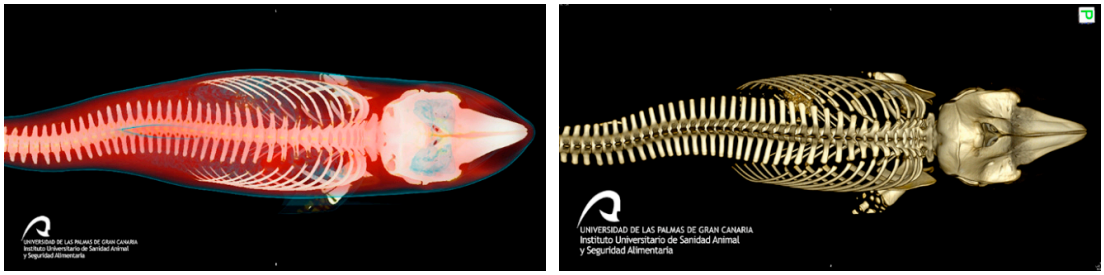
It was possible to observe that every single result obtained for CK was high. It is important to remark the new peak of the value on day 3. This animal presented one value within the normal range on day 0 for cTnI, being the rest of the measurements elevated. As noticed in the CK, the cTnI also presented a peak on day 4 and 5–1:30 h after euthanasia. The animal demonstrated higher BUN values and lower creatinine level than normal values.

3.2. Gross Results

The 105 kg juvenile male, measured 205.5 cm, had a very poor body condition, and displayed several lacerations, distributed in a multifocal manner, on the rostrum, dorsal and pectoral fins, tail flukes and ventral part of the body, attributed to live-stranding. External and internal signs of a spinal lateral curvature (S-shape) were identified at the level of the caudal peduncle (Figure 2).



(**a**) (**b**)



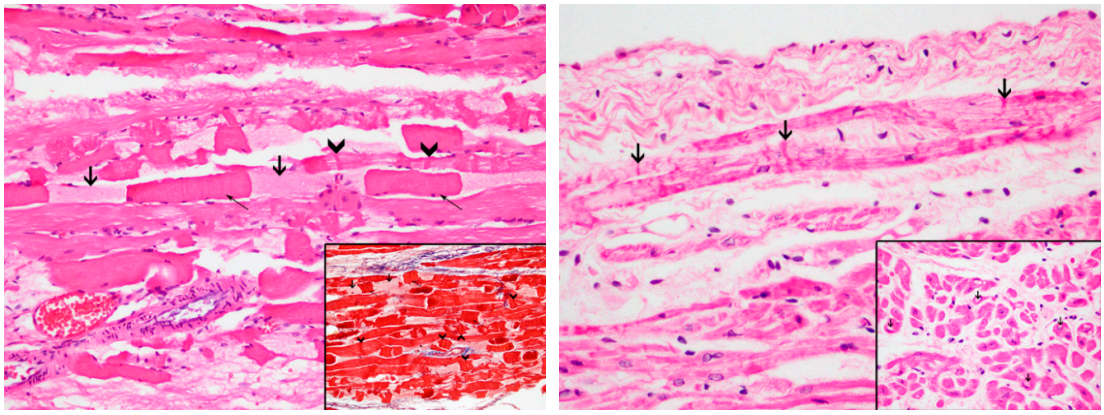
(**c**) (**d**)

Figure 2. Animal caudal peduncle scoliosis: (**a**) Caudal view on the necropsy table; (**b**) Dorsal plane of the entire body, with computer tomography (CT); (**c**) Dorsal view of the entire body, using CT, bones in white, muscle in red, and skin in blue; and (**d**) Dorsal view of the skeleton, with the CT.

3.3. Histopathological Results

On histopathologic and histochemical examinations, the skeletal and heart muscles presented injuries consistent with acute degenerative lesions. The *longissimus dorsi* and the *rectus abdominis* demonstrated a multifocal, severe contraction band necrosis and myonecrosis consisting of segmentary fibrilar degeneration with hyalinized eosinophilic sarcoplasm and hypercontraction (Figure 3a). The atria and ventricles displayed a multifocal, moderate degree of contraction band necrosis (Figure 3b), wavy fibers, hypereosinophilia, and cytoplasmic vacuolization (Figure 3b, inset) with pyknotic nucleus. All of the above changes were more pronounced in the subepicardial and subendocardial regions. The different heart sections also exhibited a multifocal, mild degree of infiltration of mononuclear cells in the areas with fibrillary ruptures.

The kidneys showed a multifocal, moderate congestion in the cortex and renal medulla. An amorphous and acidophilic substance was found inside the Bowman's capsule and in the medullary renal tubules.



(**a**) (**b**)

Figure 3. Histochemical techniques: (**a**) Contraction band necrosis (arrow heads), segmentary fibrilar degeneration (thick arrows) with hyalinized eosinophilic sarcoplasm and hypercontraction (thin arrows); 20x, HE. Inset: Detail of the myocytes with contraction band necrosis (arrow heads) and segmentary fibrilar degeneration (thick arrows) with hyalinized eosinophilic sarcoplasm and hypercontraction (thin arrows); 20×, Masson's trichrome technique; (**b**) Contraction band necrosis (arrows) in the cardiomyocytes; 40×. Inset: Detail of the intracytoplasmic vacuoles (arrows) of the cardiomyocytes; 60×, HE.

3.4. Immunohistochemical Results

The degenerated/necrotic muscular and heart cells showed homogenous, intrafibrillar depletion of cTnI (Figure 4a), cardiac troponin C (cTnC) (Figure 4b), and myoglobin (Figure 4c), and exhibited

immunolabelling for fibrinogen (Figure 4d). The kidneys did not exhibit any displacement/presence of myoglobin.

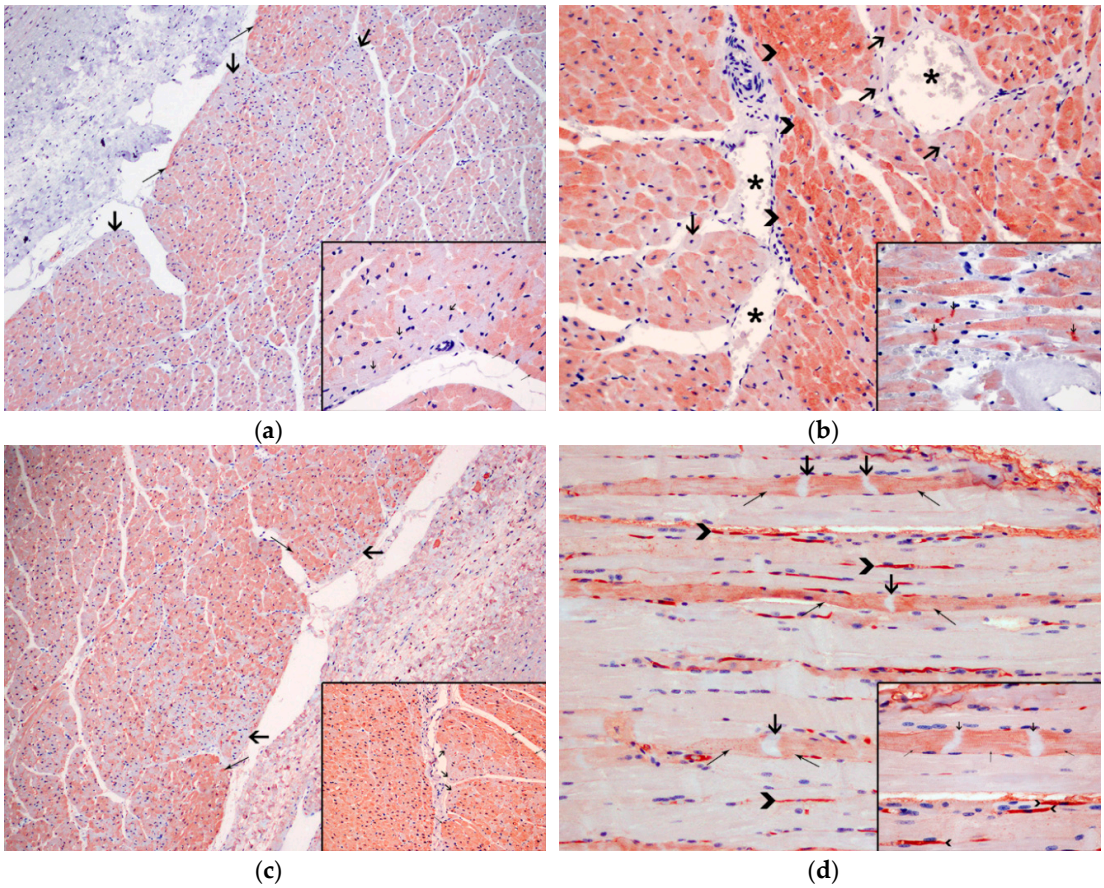


Figure 4. Immunohistochemical techniques: (**a**) Degenerated cardiomyocytes (thick arrows) present depletion of cardiac troponin I (cTnI), compared to the normal cardiomyocytes (arrow heads). 10×. Inset: Detail of the intrafibrillar depletion (thick arrows) of cTnI in the injured cardiomyocytes, in comparison with normal cardiomyocytes (thin arrows); 60×, anti-troponin I; (**b**) Necrotic cardiomyocytes (thick arrows), close to the blood vessels (*) demonstrate depletion of cardiac troponin C (cTnC), compared to the normal cardiomyocytes (arrow heads); 20×. Inset: Detail of intrafibrillar depletion of cTnC with intense immunolabelling in the contraction band necrosis (thick arrows); 60×, anti-troponin C; (**c**) Degenerated cardiomyocytes (thick arrows), show intrafibrillar depletion of myoglobin, compared to the normal cardiomyocytes (thin arrows); 10×. Inset: Detail of the intrafibrillar depletion (thick arrows) of myoglobin in the injured cardiomyocytes, in comparison with normal cardiomyocytes (thin arrows); 20×, anti-myoglobin; (**d**) Expression of fibrinogen, in the necrotic myocytes, mainly in area next to (thin arrows) the contraction band necrosis (thick arrows). Presence of fibrinogen in the blood vessels (arrow head); 20×. Inset: Detail of the immunolabelling of fibrinogen, in the injured myocytes, in the zone near to (thin arrows) the contraction band necrosis (thick arrows). Fibrinogen inside the blood vessels (arrow heads); 60× anti-fibrinogen.

4. Discussion

4.1. Discussion of the Biochemical Results

Clinico-pathological evaluation was challenging, not only because normal and/or pathological biochemical values are rarely reported for cetaceans, and in this case, particularly regarding Risso's dolphins but also due to the non-existing normal range for specific cardiac markers, such as cTnI, in the cetacean scientific database. Our biochemical analysis was compared with different mammals in published papers [23–26].

The most relevant biochemical changes described in CM in birds (i.e., wild turkeys, mallards), terrestrial (i.e., hoofed mammals), and marine wild mammals (i.e., sea otters, seals), are the CK and BUN elevation [9–12,19,21,27]. For SCMP in humans, the more common laboratories abnormalities are consistent with a small, rapid rise to above the normal levels of cTnI and/or CK [6,7].

After an injury to the muscle (both skeletal and cardiac), the serum level of CK begins to rise in 4–9 h, peaks at 24 h, and returns to baseline 48–72 h, unless a new injury or permanent damage occurs [28]. Every single result obtained is higher than the normal range published for this species (48 to 154 U/L) which is a useful indicator of both skeletal and cardiac muscle damage suffered by this animal during and after stranding, and also during the rehabilitation period [18,19,21,26,28]. Additionally, the highest value of this marker on day 3 indicates the occurrence of a new injury.

Although CK is considered a sensitive marker of myocardial damage, it is present in the skeletal muscles in high concentrations. Due to its poor specificity to detect specific heart damage, troponins have been adopted as the new gold standard for necrosis, more specifically cTnI, since it is detectable in very low amounts (0.01 μg/L) in the blood of healthy individuals [28,29]. Thus, significant elevations (≥ 0.1 μg/L) most likely reflect myocardial necrosis [28]. It is considered as cardiospecific, therefore it is used to detect heart pathologies (i.e., SCMP) [6,28,29]. Most of the injured cardiac myocytes release troponin, 3–9 h after ischemic damage, reaching its peak between 12–48 h and remains raised for up to 4–7 days, unless a new injury or permanent damage occurs [28,29].

At present, there are no published studies on *Odontoceti* that analyze specific cardiac markers to detect the resulting damage to the heart in these animals. Compared to the recommended values in humans (≤0.1 μg/L) and in dogs (≤0.03–0.07 μg/L), this animal presented one value within the normal range on day 0 (0.035 μg/L), being the rest of the measurements elevated [23–25]. All the results concur with the kinetics except on day 4 and 5 - 1:30 hours after euthanasia, which indicates the occurrence of a new stress event, leading to a new damage. In this case, pre-renal azotemia was reasonably supported by higher BUN values than the existing reference values for this species (i.e., 36 to 69 mg/dL), associating this clinical finding with various possible causes, such as compartment syndrome, heart failure, and dehydration [19,26,30]. All measurements of creatinine were below the normal range for this species (1.4 to 2.8 mg/dL) probably related to the poor body condition [26,30].

4.2. Discussion of the Gross, Histopathological and Immunohistochemical Results

The chronology of events described in this case report is concurring with previous cases of live-stranded cetaceans, being the scoliosis a common problem in these animals, occurring during the rehabilitation process secondary to lack of swimming in a compromised animal [18,31].

The histopathological findings present in animals who suffered from CM consist of acute to subacute degeneration (rhabdomyolysis) and acute renal failure associated with myoglobinuric nephrosis secondary to muscle damage. The alterations related with the SCMP present in CM are associated with morphological alterations namely acute degenerative lesions (i.e., contraction band necrosis, wavy fibers, hypereosinophilia, and cytoplasm vacuolization), vascular changes (i.e., congestion, interstitial edema, and hemorrhages) and infiltration by inflammatory cells, based on the histological analysis of myocardial tissue [4,8–11,15,19,21,27]. Baring this in mind, the skeletal muscle and kidney samples of this animal presented lesions which illustrated rhabdomyolysis and acute renal failure. In relation to the heart lesions it is possible to conclude that the animal was entering the subacute stage (1 to 3 days), described in the sequence of changes in an acute ischemic injury in the heart, since it is when lymphocytes start to appear [5]. Immediately after a vital trauma, skeletal and cardiac proteins begin to leak as a result of early cell membrane rupture, causing a quick decline in myoglobin, cTnI, and cTnC contents, along with the deposition of plasma proteins (fibrinogen) [9,10,15,32,33]. These changes were observed in our animal confirming the ante mortem lesions.

5. Conclusions

We consider this article to be an important contribution to clinical biochemistry in cetaceans. Valuable use of blood samples and finally necropsy assessment advances our understanding about the pathology in live-stranded cetaceans.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Summary of the therapeutic treatment. Table S2: Summary of the euthanasia protocol. Table S3: Summary of the immunohistochemical methodology. Table S4: Comparison between the biochemical analysis during rehabilitation (ante-mortem) and after euthanasia (post-mortem) with the normal baseline values.

Author Contributions: Methodology and formal analysis, N.C., E.S., A.F., M.Arbelo, Y.B.deQ., M.Arregui, F.C. and P.H.; writing—original draft preparation, N.C.; supervision and writing—review and editing, E.S. and P.H.; funding acquisition, A.F. and P.H. All authors have read and agreed to the published version of the manuscript.

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

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Table S1. Summary of the therapeutic treatment.

Medicine	Active Principle	Dosage [1]	Number of Dosages	Via	Days
Dexametasona Kern Pharma	Dexamethasone	0.11 mg/kg	1	Intramuscular	Day 0 (26/04/2019)
Ganadexil Enrofloxacino	Enrofloxacin	5 mg/kg	1 (each day)	Intramuscular	Day 0 (26/04/2019) Day 1 (27/04/2019) Day 2 (28/04/2019)
				Oral (diluted at 50%)	Day 3 (29/04/2019) Day 4 (30/04/2019)
Zipyran Plus	Praziquantel	10 mg/kg	1	Oral	Day 1 (27/04/2019)
Ketoisdin	Ketoconazole	5 mg/kg	1	Oral	Day 2 (28/04/2019)
Fluid therapy	Freshwater	2 liters	2	Oral	Day 2 (28/04/2019)
			1 (each day)		Day 3 (29/04/2019) Day 4 (30/04/2019)

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Table S2. Summary of the euthanasia protocol.

Medicine	Active Principle	Dosage	Number of Dosages	Via	Days	Commentaries
Tiobarbital	Sodium Thiopental	5 grams	1	Intravenous	Day 5 (01/05/2019)	The decision to euthanize the animal was taken among several veterinarians who are experts in dealing with these types of animals, one of them being the veterinarian responsible for the Wildlife Recovery Center (Gran Canaria) and veterinarians of Loro Parque. The main reason, which lead to this decision, consisted in the development of the body curvature which could not be reversible seeing that there were no adequate facilities to maintain the animal, more specifically, a pool which allow the animal to have a proper swimming pattern and not having the access to the therapeutic treatment recommend to reverse scoliosis in cetaceans in rehabilitation.

Table S3. Summary of the immunohistochemical methodology used in this study.

Antigen Retrieval	Serum	Source	Dilution	Primary Antibody	Source	Host	Type	Dilution	Secondary Antibody	Source	Dilution
Citrate buffer[1]	Swine serum[3]	Dako[4]	10%[5]	Myoglobin[6]	Abcam[10]	Rabbit	Polyclonal	1 in 200[11]	Polyclonal Swine Anti-Rabbit Immunoglobulins[15]	Dako[4]	1 in 200[16]
Citrate buffer[1]	Swine serum[3]	Dako[4]	10%[5]	Fibrinogen[7]	Abcam[10]	Rabbit	Polyclonal	1 in 50[12]	Polyclonal Swine Anti-Rabbit Immunoglobulins[15]	Dako[4]	1 in 200[16]
Citrate buffer[2]	Swine serum[3]	Dako[4]	10%[5]	Troponin I[8]	Abcam[10]	Rabbit	Polyclonal	1 in 25[13]	Polyclonal Swine Anti-Rabbit Immunoglobulins[15]	Dako[4]	1 in 200[16]
Citrate buffer[2]	Swine serum[3]	Dako[4]	10%[5]	Troponin C[9]	Abcam[10]	Rabbit	Monoclonal	1 in 250[14]	Polyclonal Swine Anti-Rabbit Immunoglobulins[15]	Dako[4]	1 in 100[17]

[1] Citrate buffer, pH 6.0, 7 minutes at 96ºC. [2] Citrate buffer, pH 6.0, 20 minutes at 96ºC. [3] Dako Swine serum (Normal) (X090110-8). [4] Dako (Glostrup, Denmark). [5] Dilution of 10 µl of serum in 90 µl of PBS. The serum is incubated in a humidity chamber for half an hour. [6] Anti-Myoglobin antibody (ab187506). [7] Anti-Fibrinogen antibody (ab34269). [8] Anti-Cardiac Troponin I antibody (ab47003). [9] Anti-Cardiac Troponin C antibody (ab137130). [10] Abcam (Cambridge, United Kingdom). [11] Dilution of 1 µl of primary antibody in 199 µl of serum at 1% in PBS (dilution of 1 µl of serum in 99 µl of PBS). The primary antibody is incubated in a humidity chamber for at least 18 hours, inside the refrigerator. [12] Dilution of 1 µl of primary antibody in 49 µl of serum at 1% in PBS (dilution of 1 µl of serum in 99 µl of PBS). The primary antibody is incubated in a humidity chamber for at least 18 hours, inside the refrigerator. [13] Dilution of 1 µl of primary antibody in 24 µl of serum at 1% in PBS (dilution of 1 µl of serum in 99 µl of PBS). The primary antibody is incubated in a humidity chamber for at least 18 hours, inside the refrigerator. [14] Dilution of 1 µl of primary antibody in 249 µl of serum at 1% in PBS (dilution of 1 µl of serum in 99 µl of PBS). The primary antibody is incubated in a humidity chamber for at least 18 hours, inside the refrigerator. [15] Dako Polyclonal Swine Anti-Rabbit Immunoglobulins/Biotinylated (E035301-2). [16] Dilution of 1 µl of secondary antibody in 199 µl of serum at 1% in PBS (dilution of 1 µl of serum in 99 µl of PBS). The secondary antibody is incubated in a humidity chamber for half an hour. [17] Dilution of 1 µl of secondary antibody in 99 µl of serum at 1% in PBS (dilution of 1 µl of serum in 99 µl of PBS). The secondary antibody is incubated in a humidity chamber for half an hour.

Table S4. Comparison between the biochemical analysis during rehabilitation (ante-mortem) and after euthanasia (post-mortem) with the normal baseline values.

Item	Time	CK (U/L)		cTnI (µg/L)		BUN (mg/dL)		Creatinine (mg/dL)	
		Study animal	Normal value for this species	Study animal	Normal value for this species	Study animal	Normal value for this species	Study animal	Normal value for this species
Ante-mortem (During rehabilitation period)	Day 0 (26/04/2019)	837.8	48 to 154 [1]	0.035	Non existing	128	36 to 69 [1]	0.7	1.4 to 2.8 [1]
	Day 1 (27/04/2019)	885.1		0.151		230		0.9	
	Day 2 (28/04/2019)	334.1		0.133		196		0.8	
	Day 3 (29/04/2019)	959.0		0.120		195		0.6	
	Day 4 (30/04/2019)	455.7		0.164		173		0.4	
	Day 5 (01/05/2019)	715.3		0.162		170		0.4	
Post-mortem (After euthanasia)	Day 5 (01/05/2019) (1:30 h after euthanasia)	843.6		0.168		171		0.4	

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CONCLUSIONS

FIRST: Cetaceans that die during and/or after stressful events such as active strandings, ship collisions and accidental interactions with fisheries, develop vascular changes and cardiac acute degenerative-necrotic injuries comparable to lesions observed in humans with stress cardiomyopathy, thus demonstrating that cetaceans are susceptible to this pathology.

SECOND: The normal reference range for serum cardiac troponin I in adult bottlenose dolphins born and maintained under human care is 0 to 0.0256 µg/L. This is the first reference in the scientific literature for blood values for cardiac troponin I in cetaceans.

THIRD: Stranded cetaceans alive and subjected to interaction with humans develop an acute stress cardiomyopathy characterized by a serum increase of cardiac troponin I, which correlates with tissue depletion of cardiac troponins I and C, in cardiomyocytes with acute degenerative changes, detected histologically.

FOURTH: The use of the clinical determination of serum cardiac troponin I and immunohistochemical analysis of cardiac troponins I and C for the diagnosis and decision making in stress cardiomyopathy in live-stranded cetaceans is validated.

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TESIS DOCTORAL

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HISTOLOGICAL, HISTOCHEMICAL, IMMUNOHISTOCHEMICAL AND BIOCHEMICAL STUDIES

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