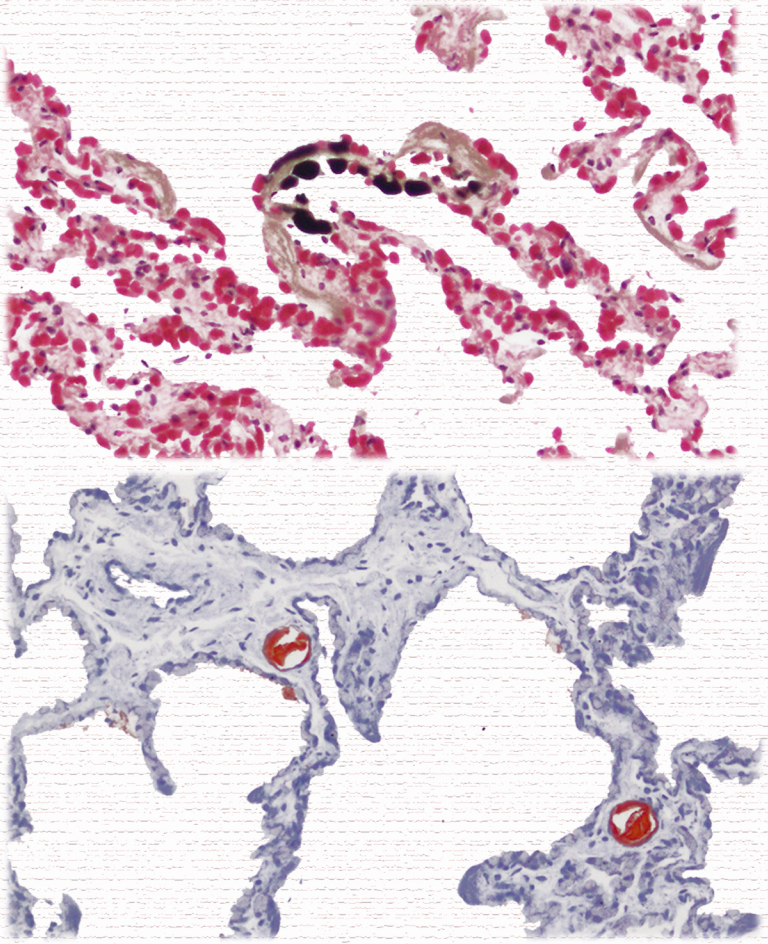
UNIVERSIDAD DE LAS PALMAS
DE GRAN CANARIA

IUSA
Instituto Universitario de Sanidad Animal

TESIS DOCTORAL

FAT EMBOLISM IN STRANDED CETACEANS

EMBOLISMO GRASO EN CETÁCEOS VARADOS



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

INFORMA,

Que la Comisión Académica del Programa de Doctorado, en su sesión de fecha / / , tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada "Fat embolism in stranded cetaceans (Embolismo graso en cetáceos varados)" presentada por la doctoranda Dª Marina Arregui Gil y dirigida por el Doctor Antonio J. Fernández Rodríguez y la Doctora Yara Bernaldo de Quirós Miranda.

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 dos mil veinte.

A mis padres

A mi hermano

A mis abuelos

Caminante, son tus huellas
el camino y nada más;
Caminante, no hay camino,
se hace camino al andar.
Al andar se hace el camino,
y al volver la vista atrás
se ve la senda que nunca
se ha de volver a pisar.
Caminante no hay camino
sino estelas en la mar.

Antonio Machado

Proverbios y cantares (XXIX)

TABLA DE CONTENIDO

1. INTRODUCTION AND OBJECTIVES

The scientific and social concern about the marine environment´s anthropogenic impact is growing in the last decades. Several studies have been published on this topic to raise public and government awareness about the necessity to implement environmentally friendly policies.

Different elements (chemical, physical, and biological) are often used as indicators of the marine ecosystem's health to assess its current state and its variation over time reliably and straightforwardly. Among the several marine living organisms, cetaceans are good bioindicators, as they are at the top of the marine trophic chain (specially odontocetes) and have long lifespans. They are also considered umbrella species, which means that they usually occupy large habitats, and their protection allows the adequate protection of many other living organisms in the ecosystem (Hoyt, 2011; Prideaux, 2003).

The main anthropogenic threats on cetacean populations worldwide include deaths due to anthropogenic sound, ship collision, plastic ingestion, or fisheries interactions, among others (e.g., Jepson et al., 2003; Laist et al., 2001; Puig-Lozano et al., 2018; Sharp et al., 2019).

Concerning anthropogenic sound, sonar activities have been linked to massive strandings and deaths of beaked whales worldwide (review in Bernaldo De Quirós et al., 2019; Cox et al., 2006; D'Amico et al., 2009). *Postmortem* studies revealed that these animals had severe, diffuse congestion and hemorrhages, mainly around the jaw fat, ears, brain, and kidneys. Moreover, gas bubble-associated lesions and fat emboli were observed in the parenchyma and within vessels of various organs, respectively (Fernández et al., 2005; Jepson et al., 2003). This pathological entity was named "Gas and Fat Embolic Syndrome" or "Decompression-like Sickness" due to the similarities with the decompression sickness syndrome described in human scuba divers (Fernández et al., 2005; Jepson et al., 2003). Decompression sickness is a syndrome caused by the formation of intra- and extravascular gas

bubbles when the dissolved atmospheric gases' tension is higher than the local absolute pressure (Vann et al., 2011).

A few years later, it was demonstrated that the chemical composition of the gas bubbles found in beaked whales stranded in temporal and spatial association with naval exercises was mainly nitrogen (N_2), the gas responsible for decompression sickness (Bernaldo de Quirós et al., 2011). Since then, several international projects and scientific publications have focused on the study of beaked whales and gas embolism. Results of these studies have revealed that beaked whales are extreme deep divers with stereotyped diving profiles (Schorr et al., 2014; Tyack et al., 2006) and suggested that behavioral and physiological responses may trigger decompression-related injuries (Bernaldo de Quirós et al., 2019; DeRuiter et al., 2013; Fernández et al., 2005; Jepson et al., 2003).

In cetaceans, gas embolism related to other causes than sonar has been described due to bycatch (Bernaldo de Quirós et al., 2013; Moore et al., 2009), to stressful predatory interactions (Fernández et al., 2017), and infection from gas-producing *Enterobacteriaceae* (Danil et al., 2014). However, the gas composition can discriminate causes as, in the two first cases, the gas composition will be dominated by N_2 and carbon dioxide (CO_2), indicating that gas was formed by decompression. In contrast, in the latter case, gas composition will be dominated by hydrogen and CO_2, indicating that gases were produced by bacteria.

Fat embolism, defined as the intravascular presence of fat that can lead to blockage of vessels (Watson, 1970), has been described in association to gas embolism, but also alone in cetaceans due to traumatic injuries consequence of vessels´ strikes or intra-interspecific interactions (Díaz-Delgado et al., 2018). Traumatic injuries caused by a live-stranding may originate or aggravate fat embolism (Bernaldo de Quirós et al., 2019).

In the last decades, ship strikes have become an anthropogenic cause of lethal and non-lethal injuries for cetaceans worldwide due to both an

increase in the ships' number and speed (Laist et al., 2001). They constitute a major threat, especially for highly endangered populations (e.g., North Atlantic right whales in the western North Atlantic (Knowlton and Kraus, 2001)), where the loss of few individuals can compromise the population survival. Research to identify the areas where cetacean strikes are frequent and evaluate the population status in those areas is essential to implement proper mitigation measures (e.g., speed restrictions) that minimize the effect of ship strikes on specific cetacean populations.

In cetaceans, traumatic interactions with individuals of the same (intraspecific) or different species (interspecific) have been described in the literature. Aggressive encounters among individuals of the same species include aggression between males (e.g., Parsons et al., 2003) or infanticides (e.g., Dunn et al., 2002; Towers et al., 2018), among others. Violent interactions with other species often occur due to prey competition, predation, or fight practices (Fernández et al., 2017; Jepson and Baker, 1998; Wellard et al., 2016). These traumatic interactions usually result in blunt traumas, internal hemorrhages, and fractured bones (Arbelo et al., 2013; Díaz-Delgado et al., 2018; Puig-Lozano et al., 2020), and occasionally in mild fat embolism (Díaz-Delgado et al., 2018; Fernández et al., 2017).

Nevertheless, the number of studies focused on cetacean fat embolism is extremely scarce compared to gas embolism, and little progress has been made in its diagnosis, forensic significance, and origin.

The present research was developed to broaden the knowledge on fat embolism alone or linked to gas embolism in cetaceans dead due to ship strikes or decompression-like sickness. Its specific objectives were:

1. To assess if the presence of fat emboli within the lung vessels is useful diagnostic evidence to assess *antemortem* ship strikes in sperm whales of the Canary Islands.

2. To develop a quantitative and objective method to determine histologically fat emboli abundance in the lungs.
3. To test and compare the efficiency of different histochemical techniques for fat embolism diagnosis.
4. To analyze the lipid composition of cetacean lung tissue with two main sub-objectives: 1) to provide a baseline of standard lung lipid composition and 2) to increase the knowledge on fat emboli lipid composition by comparing lipid composition of lungs positive and negative to fat embolism.

2. STATE OF THE ART

2.1. LIPIDS

Lipids are a heterogeneous group of organic compounds soluble in organic solvents as chloroform or acetone, and insoluble in water (Pond, 1998). They are classified in two major groups: non-polar lipids (acylglycerols, waxes, sterols such as cholesterol (CHO), sterol esters (SE), and free fatty acids (FFAs)), which are entirely hydrophobic; and polar lipids (glycerophosphatides and sphingosylphosphatides), which are amphipathic lipids meaning they have a hydrophilic and a hydrophobic end (Guschina and Harwood, 2009; Pond, 1998).

Lipids are present in all living organisms playing different and essential functions, including structural, metabolic, and endocrine roles. Among their essential functions, they form part of cell membranes (polar lipids and sterols), act as storage products to provide energy and insulation (triacylglycerols (TAGs) and waxes), they function as precursors of different substances (e.g., steroid hormones), and they participate as intermediates (or intermediates' precursors) in several cell signaling pathways (Guschina and Harwood, 2009; Pond, 1998).

As part of organisms, lipids can be involved in a vast number of pathologies as well. In many cases, proper histochemical identification of lipids concerning pathological conditions is crucial for an accurate diagnosis. Common pathologies involving lipids in humans include atherosclerosis, fatty liver, or cancer (Marinetti, 1990; Watson and De Meester, 2016). Another common pathology involving lipids, especially related to traumatic injuries, is fat embolism, which has been assessed in human and animal science. Fat embolism in cetaceans has been described and linked to various pathological entities and is the focus of the present thesis; therefore, it will be described in detail in the following sections.

2.2. FAT EMBOLISM

2.2.1. DEFINITION OF FAT EMBOLISM

Fat embolism is defined as the presence of fat globules in blood vessels, frequently leading to small-diameter vessels' mechanical obstruction (Hulman, 1995; Watson, 1970). The usual size of these fat particles ranges from 10 to 100 micrometers (μm). They are initially arrested in the lungs, where the small pulmonary microvasculature (< 20 μm in diameter) blockages their path, acting as a filter to protect the systemic circulation (Watson, 1970). Small fat droplets may pass through the lung microvasculature and arrive at the systemic circulation embolizing the brain, kidney, liver, or skin, among other organs. The patent foramen ovale (Pell et al., 1993) and arteriovenous shunts (Riding et al., 2004) are other paths that allow fat droplets to reach the systemic circulation.

Fat embolism has been widely studied in humans and experimental models using animals. Thus, in the present doctoral thesis, different aspects of fat embolism, including causes, incidence rate, clinic, and morphologic diagnosis, will be explained first based on human literature to later focus on fat embolism in cetaceans.

2.2.2. HISTORY OF FAT EMBOLISM

The first reference to fat embolism dates back to 1669 when Lower of Oxford described fat embolism in dogs that were intravenously injected with milk (Lower, 1669). In 1862, Zenker was the first to report post-traumatic embolism. He described the presence of fat emboli in the pulmonary capillaries of a railway worker who died due to a crush injury that affected the chest and the abdomen. Despite the several fractured ribs, Zenker suggested that the fat emboli's origin was the content of a damaged stomach entering the bloodstream through torn hepatic veins (Zenker, 1862).

A few years later, in 1866, Busch described for the first time the association between fat embolism and bone marrow injuries (Busch, 1866) by injecting cinnabar into the bone marrow and finding this substance in the lung after fracturing the bone. He also described that the time of the emboli's appearance is within minutes (min) after a fracture or marrow trauma. However, it was not until 1873 that Von Bergmann diagnosed a clinical disorder called fat embolism syndrome (FES) for the first time in a live human. His patient, who fell from a high roof suffering a comminuted femoral fracture, presented similar symptoms to those previously described in cats infiltrated with intravenous injections of oil. After his patient died, the autopsy revealed a massive pulmonary fat embolism (Bergmann, 1873).

Since these first discoveries, many articles and case reports about fat embolism and FES have been published, including causes, incidence, pathogenesis, clinical signs, treatment, and histological evidence.

2.2.3. CAUSES AND INCIDENCE OF FAT EMBOLISM

Traumas, especially to long bones and pelvis, or surgical traumas (e.g., joint reconstruction) constitute over 90% of the cases that lead to fat embolism development in humans. The remaining percentage has a non-traumatic origin (e.g., pancreatitis or diabetes mellitus) (Table 2.1) (Glover and Worthley, 1999; Gupta and Reilly, 2007; Serota, 1984). Fat embolism is a common and usually asymptomatic finding that appears in the majority of patients (> 80%) with traumatic injuries (Palmovic and McCarroll, 1965), which only very infrequently leads to FES.

Table 2.1. Conditions associated with fat embolism (Gupta and Reilly, 2007).

Trauma-related
Long-bone fractures
Pelvic fractures
Fractures of other marrow containing bones
Orthopedic procedures
Soft tissue injuries (e.g., chest compression with or without rib fractures)
Burns
Liposuction
Bone marrow harvesting and transplant
Non-trauma related
Pancreatitis
Diabetes mellitus
Osteomyelitis and panniculitis
Bone tumor lyses
Steroid therapy
Sickle cell hemoglobinopathies
Alcoholic (fatty) liver disease
Acute decompression sickness
Sepsis
Cyclosporine therapy

Fat embolism severity tends to be higher when fractures involve long bones, the pelvis or the spine (Fulde and Harrison, 1991; LeQuire et al., 1959; O'Higgins, 1970; Watson, 1970); and it has been related to the multiplicity of the fractures (Emson, 1958; Sevitt, 1962). Fat embolism occurs very rapidly in severe injuries, even in cases where death occurs immediately after the trauma (Bierre and Koelmeyer, 1983; Emson, 1958; Hiss et al., 1996). In fact, in forensic sciences, the presence of pulmonary fat emboli is a reliable evidence to assess the *antemortem* injury, since the cardiac function is needed, even for a short time before death, to allow the circulation of fat droplets to the lungs (Armstrong et al., 1955; Mason, 1962; Saukko and Knight, 2004). Moreover, fat embolism and lung edema extension have been positively related to the circulation persistence, which means that the longer the circulation remains before death, the higher the extent of both lesions (Mason, 1968).

2.2.4. CLINICAL SYNDROME

2.2.4.1. Fat embolism syndrome

The FES is defined as clinical signs and symptoms derived from the systemic presence of fat emboli within the microcirculation. It is a multi-organic disorder that primarily affects the lungs, brain, and skin (e.g., Bulger et al., 1997; ten Duis, 1997). Its mortality is estimated to be between 5-15% (Fulde and Harrison, 1991).

FES's overall occurrence is low; between 1-2% of patients with a long bone fracture present it (Müller et al., 1994). Its prevalence increases with the number of fractures involved, and it may reach 5-10% in patients with bilateral or multiple bone fractures (Peltier et al., 1974). It appears more frequently associated with long-closed bone and pelvic fractures (Peltier et al., 1974) rather than open fractures (Collins et al., 1968). Atraumatic causes are much less likely to lead to FES than traumatic ones (Gupta and Reilly, 2007). FES rarely occurs in children as their bone marrow is composed mainly of hematopoietic tissue instead of fat. Also, the fats present in children's bone marrow are palmitin and stearine, which are less likely to produce fat embolism compared to olein, present in an adult's bone marrow (James, 1950).

FES is characterized by an asymptomatic period with symptoms commonly appearing between 48 to 72 hours after injury. FES major symptoms following Gurd (1970) and Gurd and Wilson's (1974) criteria are still taken as a reference to diagnose FES (Table 2.2). These are:

a) Respiratory distress, due to the lodging of fat particles within the pulmonary vasculature. This symptom is the first to appear, and it is characterized by dyspnea (difficulty in breathing), tachypnea (abnormally rapid breathing), a decrease in the oxygen (O_2) partial pressure, and an increase in CO_2 partial pressure leading to hypoxia (insufficient O_2 supplies for life functions).

b) Cerebral symptoms, as a consequence of cerebral embolism and hypoxia. Nevertheless, concerning hypoxia, it should be highlighted that cerebral signs have been described in patients with no or little pulmonary involvement (Findlay and DeMajo, 1984). These symptoms usually include confusion, drowsiness, aphasia (language alteration), and apraxia (non-coordinated movements). In the most severe cases, they can lead to a coma.
c) Petechial rash, consisting of red or purple spots in the skin or mucous membranes due to the rupture of dermal capillaries and erythrocyte extravasation caused by embolization of those vessels. They can be found in the axillae, in the anterior part of the thorax and neck, in the conjunctivae, and in the mouth mucous membranes.

Other minor symptoms usually appear as well (Table 2.2). Gurd (1970) determined that at least one major and four minor criteria should be present to diagnose FES. However, the lack of agreement in the number of symptoms that need to be present and the absence of specific diagnostic laboratory tests hinders an accurate and early diagnosis, often relying on the clinicians' expertise. Imaging techniques as computed tomography (CT) of the chest (e.g., Newbigin et al., 2016) or magnetic resonance imaging (MRI) of the brain can help to reach a final diagnosis (e.g., Parizel et al., 2001).

FES has no specific treatment, being prevention, early diagnoses, and supportive care, the main tools that will allow most of the patients to recover fully within 2 to 4 weeks. An early immobilization of fractures has demonstrated to reduce the incidence of FES significantly. Mechanical ventilation in hypoxemic patients or fluid resuscitation in hypovolemic ones must be provided (Baker, 1976; Gurd, 1970; Newbigin et al., 2016; O'Higgins, 1970).

Table 2.2. Gurd's criteria to diagnose FES.

Major criteria
Petechial rash
Respiratory symptoms with radiographic changes
Cerebral signs unrelated to head injury
Minor criteria
Tachycardia < 110 bpm
Pyrexia < 38.5 °C
Retinal changes (fat or petechiae)
Renal abnormalities (oliguria, anuria, or lipiduria)
Acute drop in hematocrit or platelet values
High erythrocyte sedimentation rate
Fat globules in sputum

2.2.4.2. Fulminant fat embolism

This form of FES is rare, being patients who have suffered multiple fractures the most susceptible. It has a high mortality, and it develops within a few hours after injury. It progresses rapidly and causes death in the first 12 hours in the majority of the cases. It is caused by an acute cardiovascular and pulmonary obstruction produced by the release of a large amount of intravascular fat, followed by right heart failure. Platelet aggregation and the release of vasoactive and thrombogenic substances contribute to pulmonary hypertension and edema, exacerbating the pulmonary mechanical obstruction (Fulde and Harrison, 1991; Glover and Worthley, 1999; Hagley, 1983).

2.2.5. PATHOGENESIS OF FAT EMBOLISM

Although fat embolism's pathogenesis remains unclear, and there is a lack of agreement among authors, there are two main explanations for fat embolism's origin in the literature: the mechanical and the biochemical explanations. However, both mechanisms are not exclusive and may co-occur, or one of them may give rise to the other.

2.2.5.1. Mechanical explanation

In this explanation, the source of fat emboli is the bone marrow or the adipose tissue. Fat droplets may be released by the disruption of fat cells from the bone marrow of fractured bones or damaged fatty tissues entering the bloodstream through torn venules in the injury or fracture site due to a higher intramedullary than venous pressure. These droplets will finally arrive at the lungs, where they usually get trapped within the pulmonary microvasculature (Fig.2.1) (Gauss, 1924).

This explanation does not give reasons for the 24 to 72-hour delay in FES development. However, it is supported by:

a) The presence of bone-marrow cells in lung sections of dead patients as a consequence of traumatic injuries (Bhaskaran, 1969).
b) The same fatty acid (FA) composition of the bone marrow and the intravascular fat emboli in lung biopsies of traumatized dogs (Adar, 1972; Kerstell, 1971).
c) The efficiency of tourniquet application to the fractured limb keeps fat within it, preventing fat from reaching systemic circulation (Peltier, 1956).

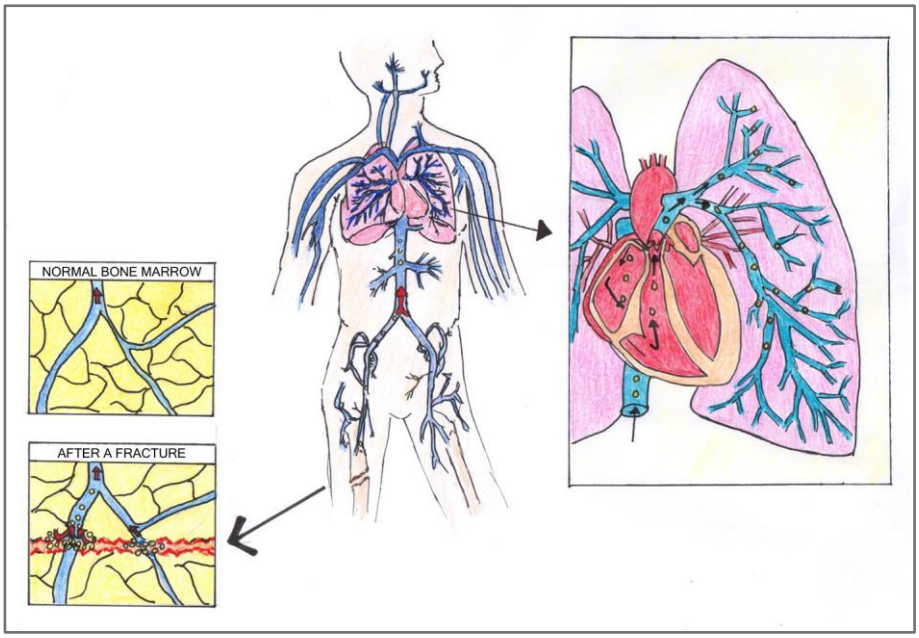


Fig. 2.1. Mechanical explanation of fat embolism.

The higher degree of fat embolism severity when bone fractures are involved may be explained by the osseous attachments of the veins within the marrow cavity. The attachments prevent veins from collapsing and allow fat to enter the venous system relatively easily, contrary to soft tissues' veins (Gauss, 1924).

2.2.5.2. Biochemical explanation

The first biochemical explanation was proposed in 1927 by Lehmann and Moore and highlighted the participation of biochemical mechanisms in FES development (Lehman and Moore, 1927). Among the different biochemical mechanisms that have been proposed, the most widely accepted explanations can be divided into toxic and obstructive, although both may co-occur (Levy, 1990).

The toxic explanation proposes that following trauma, systemic inflammation, or sepsis, FFAs are released at the trauma site or generated in the lung due to the pneumocyte hydrolyzation of fat globules. These free FAs are toxic and damage the pneumocytes and endothelial cells, causing edema and hemorrhages. They may also lead to diffuse alveolar wall damage and to adult respiratory distress syndrome (Levy, 1990; ten Duis, 1997).

The obstructive explanation proposes that following trauma, systemic inflammation, or sepsis, lipids released tend to agglutinate due to acute-phase proteins' action. Lipid agglutination leads to embolization and occlusion of the lung microvasculature (Levy, 1990; Rahman et al., 2017). Circulating chylomicrons may coalesce as well due to acute-phase proteins. Chylomicron aggregation may be crucial in non-traumatic processes, like pancreatitis, in which patients show elevated levels of C-reactive protein, which can induce calcium-dependent lipid aggregation (Hulman, 1988a; Hulman, 1988b). This obstructive mechanism may explain the development of fat embolism/FES in non-traumatic situations.

2.2.6. HISTOCHEMISTRY

Lipid biochemistry is frequently done on fresh (unfixed) frozen tissues. However, working with fresh tissue is challenging or not always possible, as rapid freezing is essential to avoid ice crystal formation and the subsequent damage to the tissue structure (Bancroft, 1975; Carriel et al., 2017).

Tissues can also be chemically fixed. This fixation preserves the structure of the living tissue as much as possible before performing any histochemical technique. Nowadays, there are numerous fixatives, and the choice of fixative to be used should be taken following the tissue elements that we want to demonstrate. Formaldehyde is a common-used fixative that does not react with lipids (it will not fix them either remove them) and, thus, can be used for lipid demonstration. Fixatives containing alcohol should be avoided as lipids are soluble in alcohol and wash them out of the tissue. Once the tissue

has been fixed, it can be embedded in paraffin wax or be cooled down to obtain frozen tissue sections that can be sliced (Bancroft, 1975; Carriel et al., 2017).

For fat emboli demonstration, the lung has traditionally been considered the target organ for fat emboli histological detection, as emboli get easily trapped within the pulmonary microvasculature (Levy, 1990; Watson, 1970). Lung fat emboli have been demonstrated histologically in human an animal science, both in frozen and paraffin-embedded sections. In the history of fat embolism demonstration, the techniques of choice have been those specialized in staining neutral lipids (sections 2.2.6.1. and 2.2.6.2.), indicating that these types of lipids are the ones composing fat emboli (Watson, 1970).

2.2.6.1. Fat embolism demonstration in frozen sections

Different staining techniques have been used for lipid demonstration in frozen sections of fresh or formaldehyde-fixed tissues. Once lipids have been demonstrated, a counterstain is applied to color the tissue's remaining features, so they are visible under the microscope.

Solvent dyes are the most frequently used to demonstrate lipids in frozen sections. These dyes stain the lipid material due to their higher solubility in lipids than in the solvent where they are previously dissolved. They usually only get dissolved in neutral (non-polar) lipids giving a weak color to polar lipids. Among them:

a) Sudan III and IV were the first techniques used to assess neutral lipids histologically (Daddi, 1896; Michaelis, 1901), and since then, they have been used in multiple studies (e.g., Armstrong et al., 1955; Gurd, 1970; Hiss et al., 1996; Hulman, 1995; LeQuire et al., 1959; Warren, 1945; Watson, 1970). These dyes stain the lipids with a red or red-orange color.

b) Sudan Black B can stain polar lipids (phospholipids (PL)) and neutral lipids, as its amino groups react with acidic groups of compound lipids. It stains the lipids with a dark blue to black color.
c) Oil Red O, which has been and is still commonly used (Emson, 1958; Hulman, 1995; Mason, 1968; McIff et al., 2010), has been argued to give superior results than other solvent dyes (French, 1926). It stains the neutral fats with red (Fig. 2.2A; protocol in appendix 7.1.).
d) Others. Specific histochemical techniques can be used to demonstrate other lipids as PL (Baker's acid hematin) or CHO (PAN method) (Kiernan, 2015). Since none of them are considered to have a role in fat embolism, these techniques would not be explained in detail.

2.2.6.2. Fat embolism demonstration in paraffin-embedded tissues

If the tissues of interest are already fixed, or there is a lack of proper equipment to properly freeze the samples, lipid demonstration in paraffin-embedded tissues has proven to be a useful technique (Bancroft, 1975; Carriel et al., 2017).

Before embedding the tissue sections in paraffin, lipids have to be post-fixed to the tissue as they are soluble in the processing solvents (alcohols and xylene) used to dehydrate the tissue before paraffin-embedding. For this purpose, different techniques have been used:

a) Osmium tetroxide (OsO_4) post-fixation technique (Abramowsky et al., 1981) has been used for decades in light and electron microscopy for lipid fixation (Bancroft, 1975; Carriel et al., 2017; Chandler and Roberson, 2009). It reacts with the double bonds of unsaturated FAs, and they reduce it to a black insoluble precipitate, fixing lipids and providing permanent staining for them (Fig 2.2B,

protocol in appendix 7.2.). This technique offers good morphological quality, and the fat emboli appear properly stained and well defined within vessels (e.g., Cantu and Pavlisko, 2018; Castro Forns et al., 1998; Ellis and Watson, 1968; Sierra et al., 2007; Suárez-Bonnet et al., 2008). However, OsO_4 is incredibly toxic, especially its vapors, and safety measures should be strictly followed when working with this reactive (National Center for Biotechnology Information, 2005a; United Nations, 2017).

b) The chromic acid post-fixation technique is a more recent technique based on the infiltration of an emulsion of unsaturated lipids in the formaldehyde-fixed tissues. These lipids partition into tissue lipids, and later, chromic acid transforms the introduced lipids in organometallic complexes that are insoluble in organic solvents. Unlike OsO_4, chromic acid does not stain lipids, and paraffin-embedded tissues are later stained with Oil Red O (Fig 2.2C) (Tracy and Walia, 2002; Tracy and Walia, 2004). Chromic acid should be carefully handled as it is a hazardous and possible carcinogenic chemical (National Center for Biotechnology Information, 2005b).

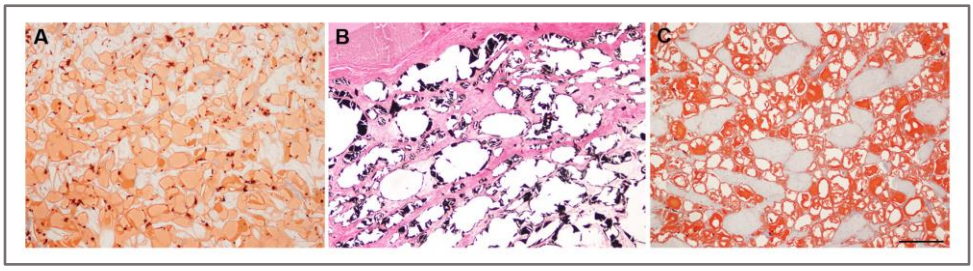


Fig. 2.2. Lipid staining techniques: A) Cetacean blubber adipocytes stained with Oil Red O in frozen sections (Bar= 200 µm), B) Adipocytes of rete mirabile demonstrated with OsO_4 (Bar= 500 µm), and C) Blubber adipocytes demonstrated with chromic acid (Bar= 200 µm).

2.2.7. FAT EMBOLISM SEVERITY ASSESSMENT

As previously mentioned, FES is diagnosed based on clinical signs and symptoms in live patients. In forensic science, fat embolism assessment must be done based on the histological demonstration of fat emboli within vascular vessels and the proper determination of its severity. Our interpretation is that the higher the number of emboli or the tissue section occupied by those emboli, the higher is the potential role that fat embolism may have had in a patient's death.

To date, there is no consensual index to grade fat embolism in pulmonary tissue. Different semi-quantitative indexes have been proposed for its gradation based first, on the number of emboli encounter in specific microscopic fields or the whole tissue section and secondly, on the form of those emboli as Mason, Sevitt, or Falzi's scales (Falzi et al., 1964; Mason, 1962; Sevitt, 1962) (Table 2.3). In 1988, Bunai and colleagues claimed that the number, the size, and the location of the emboli (the type of vessel) should be considered for a proper severity assessment (Bunai et al., 1988). In 1994, Busuttil and Hanley determined that as fat emboli seem to be randomly distributed throughout the tissue, measurements not considering the total tissue section area studied will lead to inaccurate results. Thus, they proposed a new system based on the total embolized area compared to the sample's total tissue area (Busuttil and Hanley, 1994). Later, a study carried out by Turillazzi et al. (2008) investigated different parameters to assess fat embolism severity and concluded, in agreement with Busuttil and Hanley (1994), that the most reliable parameters to grade fat embolism seemed to be the embolized tissue area related to the total tissue area of each sample.

Table 2.3. Histological semi-quantitative indexes for fat embolism's graduation in pulmonary tissue.

Semi-quantitative indexes	Area of study	Grade	Definitions
Mason (1962)	Whole tissue sample	0	No emboli seen
		1	Emboli found after some searching
		2	Emboli seen easily
		3	Emboli present in large numbers
		4	Emboli present in potentially
Sevitt (1962)	20-40 fields at 10 magnification (x)	1	Average < 1 emboli per field
		2	Average 1-3 emboli per field
		3	Average > 3 emboli per field
Falzi et al. (1964)	Every field at x25	0	No or sporadic fat droplets
		1	Drop-shaped in every x25
		2	Sausage-shaped in every x25
		3	Antler-shaped in every x25

2.2.8. FAT EMBOLI CHEMICAL COMPOSITION

Studies aiming to assess fat emboli lipid composition are very scarce in the literature despite their importance, as their composition should resemble the composition of the tissue/s where they originated, contributing to the etiopathogenic knowledge of fat embolism.

Early studies encounter the technical problem of separating plasma lipids, where fat emboli were included, from tissue lipids. In 1966, Hallgren and collaborators overcame this problem by perfusing the lung blood vessels of traumatized dogs in a retrograde direction, collecting the perfusate, and analyzing the lipids obtained using gas-liquid chromatography (Hallgren et al., 1966). This methodology was later improved by Sherr and Gertner (1974) to allow the collection of most of the fat emboli present in the perfused lung.

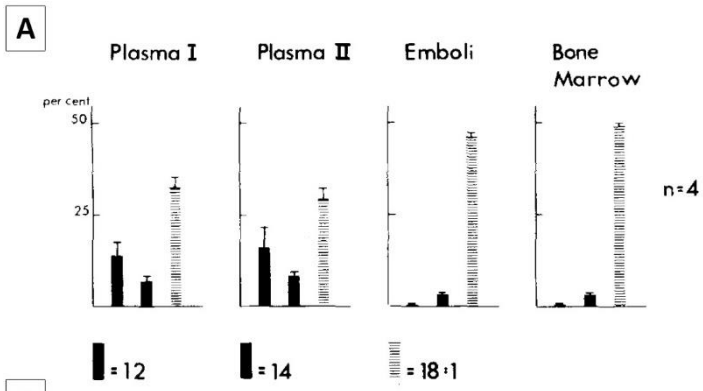
In the studies carried out by Kerstell (1971) and Sherr et al. (1974), the chemical composition of the recovered lung emboli from dogs with bilateral femur fractures showed TAGs were the predominant lipids composing fat emboli and that the FA composition of those TAGs was very similar to that of the bone marrow (Fig. 2.3). This conclusion could be established because the FA composition of adipose tissues and plasma differed (Gelin et al., 1967).

Concerning the role that chylomicrons may have in fat emboli formation, different experiments have been carried out. In one of them, dogs were fed with coconut butter or synthetic tristearate, a few hours before fractures were produced. Plasma TAG composition changed due to chylomicron formation containing the administered FAs. On the contrary, bone marrow lipid composition was not altered in the postabsorptive state and showed a very similar lipid composition to lung fat emboli, unlike plasma lipid composition, evidencing that the source of fat emboli was the bone marrow tissue (Fig. 2.3) (Hallgren et al., 1969).

In another study, dogs were fed with cream containing radioactive labeled FAs before fractures were produced, and demonstrated that no significant radioactivity was found in TAGs of bone marrow or fat emboli, contrary to plasma TAGs (Kerstell, 1969a).

Finally, in a third study, the liver and intestines, organs in charge of plasma lipoproteins' formation, were extirpated from dogs before traumatizing them. Results showed that fat emboli formation could not be prevented, demonstrating that free FAs released from the adipose tissue by trauma and re-esterified in the liver and intestines were not the origin of fat emboli formation (Kerstell, 1969b).

In conclusion, these experiments shattered the hypothesis that chylomicrons, present in the blood in the post-alimentary phase, or FFAs re-esterified to form TAGS, could be the source of fat embolism or at least contribute to them in trauma cases.



B

Fatty acid	Emboli (n = 25)	Bone Marrow (n = 25)	Plasma (n = 23)
14	3.4 ± 0.21	3.5 ± 0.06	0.4 ± 0.08
16:0	23.3 ± 0.38	23.6 ± 0.56	22.3 ± 0.49
18:0	7.6 ± 0.22	8.2 ± 0.30	8.6 ± 0.52
18:1	46.0 ± 0.80	46.1 ± 1.01	36.2 ± 1.33
18:2	7.6 ± 0.32	7.4 ± 0.44	9.7 ± 0.48
20:p	1.1 ± 0.22	0.8 ± 0.13	6.9 ± 1.02
22:p	0.3 ± 0.04	0.3 ± 0.07	3.2 ± 0.37

Fig. 2.3. A) Percentages of 12, 14, and 18:1 FAs of TAGs in plasma before fracture (plasma I) and before perfusion (plasma II), in fat emboli, and bone marrow in four traumatized dogs fed with coconut butter; B) Mean FA composition (%) of the main FA of TAGs in emboli and bone marrow in twenty-five traumatized dogs. The mean FA composition (%) in postabsorptive plasma is also given. Image from Kerstell (1971).

2.3. FAT EMBOLISM IN CETACEANS

Cetaceans moved from land to water around 55 million years ago, making their transition from terrestrial to aquatic specialists (Fordyce and de Muizon, 2001; Thewissen and Williams, 2002). For this, they developed several anatomical and physiological adaptations to meet the physical needs of an aquatic environment (Berta et al., 2015). Molecular studies have demonstrated the close relationship between cetaceans and artiodactyls (even-toed ungulates), constituting together the Order Cetartiodactyla (Price et al., 2005). Within this group, cetaceans are enclosed within the infraorder Cetacea, which is composed of two superfamilies: Mysticeti (baleen whales) and Odontoceti (toothed whales) (WoRMS, 2020).

Fat embolism has been as well demonstrated in cetaceans, although the number of publications in the existing literature is incredibly scarce (Arbelo et al., 2013; Díaz-Delgado et al., 2018; Fernández et al., 2005; Fernández et al., 2017; Jepson et al., 2003). The major pathological entities in cetaceans in which fat embolism has been described are ship strikes (Fig. 2.4A) and decompression-like sickness (Fig. 2.4B). Others, as intra-interspecific interactions or a potential live-stranding event, should be mentioned as well (Fig. 2.4C).

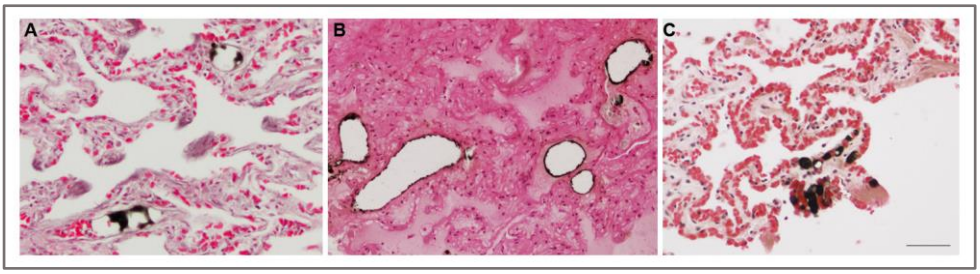


Fig. 2.4. Black stained (OsO_4-positive) fat emboli in the lung microvasculature: A) In arterioles of a struck *P. macrocephalus* calf; B) In arterioles of an adult *Z. cavirostris* with decompression like-sickness; and C) In the capillaries of an intra-interspecific traumatized adult of the species *G. griseus* (A & B: Bar= 100 µm, C: Bar= 50 µm).

2.3.1. SHIP STRIKES

In the last decades, the increase in the number and speed of the ships constitute a threat to several cetacean populations (Laist et al., 2001). Most of these vessels surpass the 15 knot-speed, over which chances of lethal injuries increase toward 100% (Vanderlaan and Taggart, 2007).

The cetacean species most affected worldwide by ship strikes are fin whales (*Balaenoptera physalus*), humpback whales (*Megaptera novaeangliae*), North Atlantic right whales (*Eubalaena glacialis*), and sperm whales (*Physeter macrocephalus*) (Van Waerebeek and Leaper, 2008). Ship strikes may compromise some cetacean species' population status in areas with high cetacean diversity and high maritime traffic overlap. Based on this overlap and reported incidents, the International Whaling Commission has established high-risk areas in which mitigation measures should focus. Some of these areas include the Arabian Sea, the Gulf of Panama, areas in the Mediterranean Sea, or the Canary Islands (Cates et al., 2016).

The usual lesions seen when a whale gets hit by a vessel are sharp or blunt trauma lesions that most commonly involve incising wounds affecting underlying tissues in the first category; and areas with hemorrhage, edema, and fractures in the second category (Campbell-Malone et al., 2008; Moore et al., 2013). In very decomposed carcasses, differentiation between *antemortem* lesions and *postmortem* changes is not always feasible. The presence of fat embolism in cetaceans may be a good indicator of *antemortem* trauma as it is in humans (Armstrong et al., 1955; Mason, 1968; Saukko and Knight, 2004), constituting valuable diagnostic evidence to assess *antemortem* ship-strike events.

In struck animals, the origin of the fat emboli encountered in the lungs is expected to be the bone marrow of fractured bones, or fat cells from damaged adipocyte-rich soft tissues (e.g., blubber) as in other traumatized animals, including humans (Bhaskaran, 1969; Kerstell, 1971; Rodríguez, 2016).

2.3.2. DECOMPRESSION-LIKE SICKNESS

Beaked whales (family Ziphiidae) have been diagnosed with an acute and systemic gas and fat embolic syndrome, similar to decompression sickness in humans, when stranding in temporal and spatial association with naval maneuvers worldwide (review in Cox et al., 2006; Fernández et al., 2005). The animals presented severe and diffuse congestion and hemorrhages in various tissues and organs, as well as bubble-associated lesions and fat embolism in the parenchyma and vessels of various vital organs (Fernández et al., 2005; Jepson et al., 2003). Decompression sickness due to natural causes has also been described in Risso's dolphins (*Grampus griseus*) (Fernández et al., 2017).

The origin of fat embolism associated with decompression sickness remains a mystery. To determine the origin of these fat emboli is extremely important for a better understanding of fat embolism's pathogenesis in decompression sickness cases. The most supported hypothesis postulates that lipid-rich tissue is disrupted by gas bubble formation within the tissue, leading to the entrance of fat emboli into the bloodstream (Hulman, 1995; Kitano and Hayashi, 1981). This hypothesis is supported by the fact that N_2 is five times more soluble in lipids than in water (Vernon, 1907; Weathersby and Homer, 1980); and that *in vivo* formation of gas bubbles has been reported in the abdominal adipose tissue of mice under hyperbaric treatments (Lever et al., 1966).

2.3.3. CETACEAN LUNGS

In cetaceans, the presence of fat emboli within the lung microvasculature constitutes a pathological finding that, considered together with other gross and microscopic findings, can lead to a final diagnosis like in human and animal science.

Cetacean lungs are located within the thoracic cavity with their base following the curvature of the diaphragm. Their costal side is adapted to the ribs' shape, and their ventrolateral side partially covers the heart (Cozzi et al., 2017). Their lungs are unilobular with an oblong to pyramidal shape (Fig. 2.5A) (Piscitelli et al., 2013).

The terminal part of the bronchial tree system in these animals differs from land mammals. In the later, terminal and respiratory bronchioles, which lead to alveolar sacs, are no longer reinforced by cartilage. On the contrary, cetaceans cartilage is present in the bronchial tree, including terminal bronchioles (Cozzi et al., 2017). The presence of cartilaginous reinforcements down to the terminal bronchioles serves two main functions. The first is to facilitate large tidal volumes (air volume inhaled and exhaled in a normal breath) and fast ventilation at the surface; the second is to provide an air storage site when alveolar sacs collapse at depth, preventing gaseous exchange. These reinforcements are aided by myoelastic sphincters located in the submucosa of bronchioles with a diameter lower than 2 mm. Myoelastic sphincters constitute a unique and characteristic feature of cetacean lungs (Fig. 2.5B) (review in Piscitelli et al., 2013).

The alveolar septa are separated by connective tissue with elastic fibers into two alveolar surfaces (Fig. 5D). Both surfaces are composed of juxtaposed blood capillaries and alveolar epithelial cells. Thus, unlike terrestrial mammals in which a single epithelium supports a single capillary bed common to two adjacent alveoli, each alveolar septum has a double capillary bed in cetaceans, meaning that capillaries are in contact with a single alveolus, allowing a higher gas exchange velocity. Macrophages are

commonly present in alveolar ducts, alveolar lumina, and in the interstice (Cozzi et al., 2017; Fanning, 1977; Henk and Haldiman, 1990; Ito et al., 1967; Piscitelli et al., 2013; Wislocki, 1929).

The alveolar septum epithelium is composed of type I and type II cells (Fig. 2.5E), similar to terrestrial mammals but lacking type III brush cells (Fanning, 1977). Type I are squamous cells, covering most of the alveolar surface, that favor gas exchange due to their high permeability. Type II are cuboidal cells containing cytoplasmatic multilamellar bodies that act as a progenitor of both type I and II in response to damage, and they produce, store, and release pulmonary surfactant in the air-liquid interface of alveoli and conducting airways (Fanning, 1977; Parent, 2015; Piscitelli et al., 2013). Lung surfactant is a substance composed of PL (90%) and proteins (10%), whose function is to reduce the air-water tension of terminal airways, preventing lung collapse (Veldhuizen et al., 1998).

2.3.3.1. Lung circulation

Like the rest of the species, cetacean's lungs have a double circulation consisting of the functional blood supply or pulmonary circulation, involved in blood oxygenation, and the nutritional blood supply or bronchial circulation, part of the systemic circulation, involved in the lungs' nourishment (Nickel et al., 1979).

In the functional blood supply circuit, the pulmonary trunk arises from the right ventricle carrying deoxygenated blood and divides into two pulmonary arteries, each of which enters a lung. Their ramifications supply lung capillaries that later drain in branches of the pulmonary vein at the alveoli intersection. These branches, carrying oxygenated blood, join each other resulting in the pulmonary veins that come out of the lungs to the left atrium (Piscitelli et al., 2013; Ward and Nicholas, 1984). Unlike veins, arteries follow the bronchial branching pattern for ramifications, as in most land mammals (Consoli, 2016). In a study carried out in striped dolphins, vascular density

was macroscopic and microscopically similar at different lung levels (Consoli, 2016).

In the nutritional blood supply circuit, the arterial blood supply reaches terminal bronchioles through ramifications and drains to the systemic circulation, although they can also drain to pulmonary venules through shunts. Its contribution to lung blood supply is much lower than the pulmonary circulation (Parent, 2015).

In cetaceans, blood vessels have a similar structure than inland mammals, with three distinctive layers: tunica intima, tunica media, and tunica externa, except that their veins have few or no valves. Arteries have a thicker wall than veins, especially the tunica media, a layer composed of smooth muscle cells and elastic fibers, as they carry blood ejected from the heart at high pressures (Fig. 2.5C). Arteries present a small and rounded lumen and can be divided into elastic and muscular. Elastic arteries are closer to the heart and have abundant elastic fibers to expand and recoil. Muscular arteries are further from the heart and are not exposed to high pressures as the elastic arteries, explaining why they have decreased the number of elastic fibers in their tunica intima and increased the smooth muscle present in their tunica media, to allow vasoconstriction. Finally, arterioles are much smaller arteries, in which the three layers, although present, have reduced their thickness, especially the tunica media, which is reduced to one or two smooth muscle cells in thickness (Betts et al., 2017). They lead to capillaries composed of an endothelial layer (tunica intima) covered by a basement membrane (Fanning, 1977). Finally, capillaries join to form venules, which in turn joint to form veins. Both are thin-walled vessels with large and irregular lumens (Fig. 2.5C) (Betts et al., 2017).

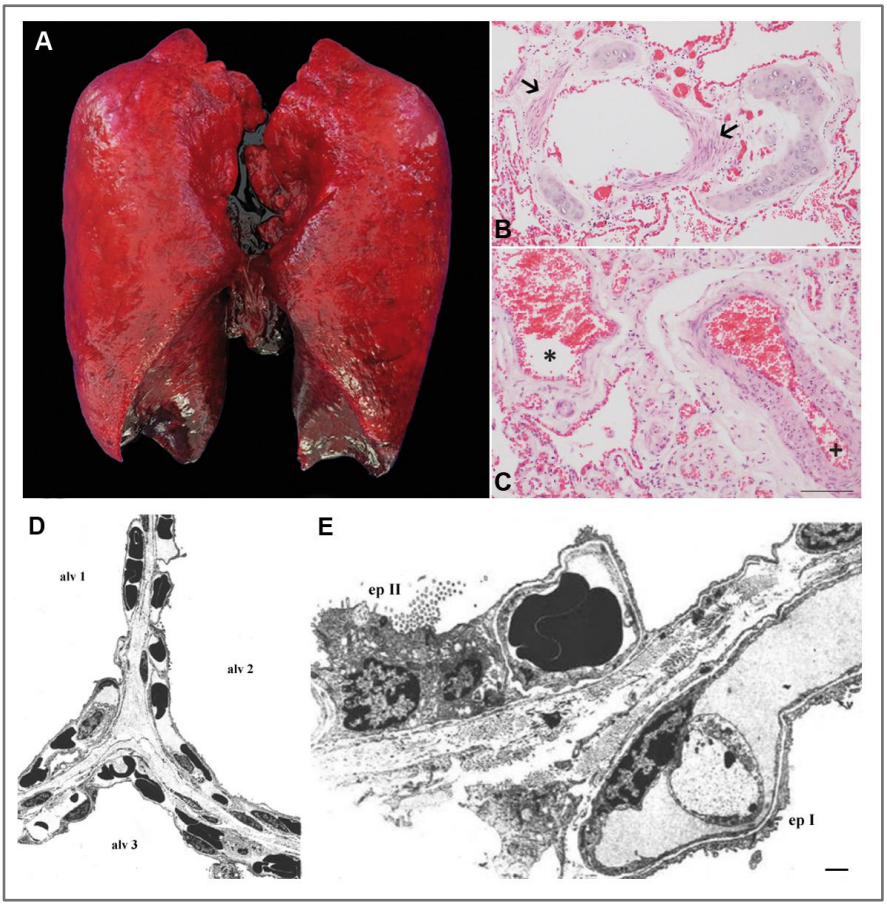


Fig. 2.5. A) Dorsal view of the lungs of an adult *S. coeruleoalba*; B) Myoelastic sphincters in a bronchiole´s submucosa (arrows); C) Detail of a pulmonary artery (plus sign) and a pulmonary vein (asterisk) (B & C: HE, Bar= 200 µm); D and E) Electron micrographs of lung sections from Fanning (1977) (Bar = 2 and 1 µm, respectively): D) Alveolar septa separating three alveoli and E) Type I and type II cells in the alveolar septum.

2.3.4. LIPIDS IN CETACEAN'S TISSUES

Cetaceans have a specialized hypodermis rich in lipids, called blubber. The blubber is composed of adipocyte cells supported by a network of collagen and elastin fibers. Its main functions are to provide thermal insulation, store energy in a lipid form, control buoyancy, and streamline the body shape (Iverson, 2009).

In most cetaceans, the blubber is mainly composed of TAG (three FAs attached to a glycerol backbone) containing FAs, usually between 14-24 carbons. TAGs are the main form of metabolic lipid storage in mammals and can be rapidly mobilized (Pond, 1998). In most species, the blubber is stratified, presenting higher concentrations of dietary and unsaturated FAs in the inner layers, and endogenous and saturated FAs in the outer layers (Koopman, 2007; Krahn et al., 2004). However, cetacean species included in the families Ziphiidae (beaked whales), Kogiidae (pygmy and dwarf sperm whales), and Physeteridae (sperm whales) constitute an exception to this as most of their blubber lipids are wax esters (WE; FA esterified to fatty alcohol (FAlc)) (review in Koopman, 2018), not known to function as a metabolic energy source (Koopman, 2007). WEs proportions in these species range from 62% in the pygmy sperm whale to over 95% in the sperm whale and some ziphiid species (review in Koopman, 2018). It has been suggested that blubber composed of WE may increase thermal insulation (Bagge et al., 2012).

Other adipose deposits in cetaceans' bodies are the acoustic fats (melon and mandibular fats), which are specialized cranial fat bodies involved in the sound transmission and reception (Norris, 1968). The melon is involved in transmitting sound and is located in the forehead region, sitting in the skull basin. The echoed sound waves are transmitted to the ear bones through the mandibular fats (external and internal). Acoustic fats of all cetaceans show a high lipid percentage (usually >80% wet weight), are composed of a mixture of TAGs and WEs and are believed to have an endogenous origin (Koopman et al., 2003; Koopman et al., 2006; Morii and Kaneda, 1982). Shorter length

endogenous iso-acids called branched-chain FAs (BCFAs), dominate these tissues as part of TAGs and WEs, derived from the catabolism of branched-chain amino acids (Morii and Kaneda, 1982). Without considering river dolphins, due to the scarce data available, the most abundant BCFAs are *i*-12:0 in the family Ziphiidae and *i*-5:0 in the families Delphinidae (dolphins), Phocoenidae (porpoises), and Monodontidae (beluga and narwhal). Finally, in species from the Physeteridae and Kogiidae families, BCFAs are present in trace amounts in acoustic fats, and they are dominated by saturated straight FAs (10:0, 12:0, 14:0, and 16:0). Among the FAlcs present in WEs, 16:0alc and *i*-16:0alc comprised the majority (50-75%) for all the families (review in Koopman, 2018). See lipid analysis´ protocol in appendix 7.3.

Little is known about the lipid content and lipid composition of the rest of the organs and tissues in cetaceans. Organs such as skeletal muscle, lung, heart, kidney, liver, stomach, and intestine, showed low lipid percentages ranging between 1-4%, and others as the brain showed higher percentages (4-8%) (Kawai et al., 1988; Morii, 1980; Storelli et al., 1999; Yordy et al., 2010). In most of these organs, PL were the dominant lipid class constituting cell membranes (Kawai et al., 1988). Fat deposits are present in the bone medullae of cetaceans as in terrestrial mammals and have been attributed to constitute up to 50% of the cetacean skeleton wet-weight (Rommel et al., 2006). In the studies carried out by Honda and colleagues in striped dolphins, the skull and caudal vertebra showed the highest lipid contents (15-25%) (Honda et al., 1984a; Honda et al., 1984b). However, cetacean bones have a cancellous structure, and unlike terrestrial mammals, lack a visible marrow cavity complicating bone marrow biopsies and the study of its lipid composition (De Buffrenil and Schoevaert, 1988; Itou et al., 2010).

2.3.5. NITROGEN SOLUBILITY IN CETACEAN ADIPOSE TISSUES

In cetaceans, studies on N_2 solubility in the blubber of different odontocete species demonstrated that N_2 solubility is higher when WE are present in the blubber, being the highest solubility values for odontocete species with wax-dominated blubber (e.g., ziphiids and kogiids) (Koopman and Westgate, 2012; Lonati et al., 2015). In acoustic fats, larger quantities of shorter branched-chain FAs and FAlcs, present in most odontocete species, together with a higher WE content, increase N_2 solubility (Lonati et al., 2015).

Among these fat compartments, different authors pointed out to the acoustic fats to explain the origin of fat emboli (Costidis and Rommel, 2016; Fernández et al., 2005). Evidence supporting acoustic fats' implication in the origin of fat emboli is their higher N_2 solubility compared to blubber due to larger quantities of short BCFAs (Lonati et al., 2015), the frequent observation of hemorrhages in these tissues in stranded individuals presenting decompression-like sickness (Fernández et al., 2005), and finally, contrary to what it was previously thought, the extensive acoustic fats´ vascularization, with veins more present than arteries, providing evidence for an important role of these tissues in N_2 exchange while diving (Costidis and Rommel, 2012).

2.4. SUMMARY OF PAPERS

To achieve the objectives of this doctoral thesis, the following studies, resulting in three publications indexed in the Journal of Citation Reports, were performed:

Publication I: Fat embolism and sperm whale ship strikes

In the Canary Islands, the sperm whale is the cetacean species most affected by ship strikes. Since 2000, over 57% of the sperm whales stranded in these islands showed evidence of a ship strike. However, most of them presented an advanced decomposition state that hindered the differentiation between *antemortem* and *postmortem* trauma. In advanced decomposed carcasses, histological fat emboli detection may help assess severe *in vivo* trauma in sperm whales, as it has already been done in other species.

In this study, fat emboli´s presence was evaluated in the lung of 24 sperm whales stranded in the Canary Islands, 16 of them with evidence of ship strike, using the OsO_4 histochemical technique. The area occupied by fat emboli in the lung sections studied was also determined for each animal. Results indicate that OsO_4-positive fat emboli were present in the lung of 13 out of the 16 sperm whales with signs of ship strike studied. This finding indicates that (1) fat emboli detection is a valuable diagnostic proof for *in vivo* trauma, even in decomposed tissues kept in formaldehyde for long periods, and (2) that in the study period, at least 81% of the sperm whales with signs of ship strike were alive at the moment of the strike and died subsequently to it.

Publication II: Comparison of three histological techniques for fat emboli detection in lung´s cetacean tissue.

In the last decades, various techniques have been used to demonstrate histologically fat emboli, being the reactive OsO_4 commonly used. However, OsO_4 is an extremely toxic reactive that must be used in well-equipped laboratories following strict safety measures. This study aimed to compare qualitatively and quantitatively the OsO_4 capacity to demonstrate fat embolism with two other techniques that have already been used for fat emboli detection and that are less toxic: chromic acid and oil Red O frozen techniques.

For that, the lungs of eight sperm whales that were positive to fat embolism were tested using the three techniques, being the first time the chromic acid technique was tested in cetaceans. Results showed that the three techniques were suitable to detect histologically fat embolism in cetaceans, even in tissues presenting advanced autolysis and stored in formaldehyde for years. There were no quantitative differences in fat emboli detection among the three techniques. Qualitatively, chromic acid showed a fat emboli-staining quality superior to the frozen section technique, and comparable to the OsO_4, even higher when considering the emboli center. We concluded that the chromic acid technique constitutes an excellent alternative to OsO_4 due to its slightly lower toxicity, equivalent fat emboli detection capacity, and significantly lower economic cost.

Publication III: Lipids of lung and lung fat emboli of the toothed whales (Odontoceti)

Knowledge of standard lung tissue lipid composition is extremely scarce in cetaceans. However, it is needed to assess pathologies involving lipids, as it is the case of fat embolism. Thus, in the present study, lung tissue's lipid composition was determined in seven odontocete cetacean species first. Secondly, the lipid composition of animals positive and negative to lung fat embolism from the same species was compared to determine the composition of fat emboli and understand fat embolism's etiopathogenesis.

Results of lipid content, lipid classes, and FAs were similar to those reported in other vertebrates, indicating that lung tissue lipid composition is conserved in the animal kingdom. When assessing fat embolism, non-significant differences could be established between lipid content, lipid classes, and FA composition between positive and negative animals, but an unidentified peak found in the struck whales' chromatogram that merits further investigation.

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3. SCIENTIFIC PUBLICATIONS

frontiers in Marine Science

ORIGINAL RESEARCH
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Fat Embolism and Sperm Whale Ship Strikes

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Strikes between vessels and cetaceans have significantly increased worldwide in the last decades. The Canary Islands archipelago is a geographical area with an important overlap of high cetacean diversity and maritime traffic, including high-speed ferries. Sperm whales (*Physeter macrocephalus*), currently listed as a vulnerable species, are severely impacted by ship strikes. Nearly 60% of sperm whales' deaths are due to ship strikes in the Canary Islands. In such cases, subcutaneous, muscular and visceral extensive hemorrhages and hematomas, indicate unequivocal *antemortem* trauma. However, when carcasses are highly autolyzed, it is challenging to distinguish whether the trauma occurred *ante-* or *post-mortem*. The presence of fat emboli within the lung microvasculature is used to determine a severe "*in vivo*" trauma in other species. We hypothesized fat emboli detection could be a feasible, reliable and accurate forensic tool to determine ante-mortem ship strikes in stranded sperm whales, even in decomposed carcasses. In this study, we evaluated the presence of fat emboli by using an osmium tetroxide (OsO_4)-based histochemical technique in lung tissue of 24 sperm whales, 16 of them with evidence of ship strike, stranded and necropsied in the Canaries between 2000 and 2017. About 70% of them presented an advanced autolysis. Histological examination revealed the presence of OsO_4-positive fat emboli in 13 out of the 16 sperm whales with signs of ship strike, and two out of eight of the "control" group, with varying degrees of abundance and distribution. A classification and regression tree was developed to assess the cut off of fat emboli area determining the high or low probability for diagnosing ship-strikes, with a sensitivity of 89% and a specificity of 100%. The results demonstrated: (1) the usefulness of fat detection as a diagnostic tool for "*in vivo*" trauma, even in decomposed tissues kept in formaldehyde for long periods of time; and (2) that, during this 18-year period, at least, 81% of the sperm whales with signs of ship strike were alive at the moment of the strike and died subsequently. This information is highly valuable in order to implement proper mitigation measures in this area.

Keywords: sperm whale, *antemortem*, fat embolism, Canary Islands, ship strike

INTRODUCTION

Strikes between vessels and cetaceans ("ship strikes") have become an issue of concern in the last decades due to an increase of the number and speed of ships (Laist et al., 2001). Reports of ship strikes have been published worldwide with fin whales (*Balaenoptera physalus*), humpback whales (*Megaptera novaeangliae*), North Atlantic right whales (*Eubalaena glacialis*) and sperm whales (*Physeter macrocephalus*) being the most affected species (Van Waerebeek and Leaper, 2008). Areas with high cetacean diversity and high maritime traffic overlap have been identified as hot spots as ship strikes may compromise the population status of some cetacean species in those areas. In Europe, these areas include the Mediterranean Sea (Panigada et al., 2006; Frantzis et al., 2019), the Strait of Gibraltar (de Stephanis and Urquiola, 2006) and the Canary Islands (Carrillo and Ritter, 2010).

The Canary Islands form a Spanish archipelago of seven main volcanic islands, located in the north-west of Africa. It is one of the richest areas for cetacean biodiversity in the Northeast Atlantic, with 30 species identified, the sperm whale among them (Tejedor and Martín, 2013). Sperm whales are present year round in Canarian waters, with higher numbers in spring and autumn due to seasonal migrations (André, 1997). They are listed as vulnerable by the International Union for Conservation of Nature (Taylor et al., 2008), and are the most affected species by ship strikes in Canarian waters (Arbelo et al., 2013; Díaz-Delgado et al., 2018). Some factors proposed to explain the susceptibility of sperm whales to ship strikes are: (1) long periods at the surface for socialization or resting after prolonged dives (Whitehead and Weilgart, 1991; André, 1997; Watkins et al., 1999; Watwood et al., 2006); (2) drift-dives, performed at a low-activity state, which will allow them to perform bi-hemispheric sleep, being unaware of approaching ships until being touched (Miller et al., 2008); or (3) possible loss of sensitivity to low-frequency sounds produced by ship engines in Canarian waters (André, 1997).

International but mainly inter-island ferry traffic in the Canarian waters has increased considerably in the last years including: normal ferries (15–20 knots), fast ferries (21–29 knots), and high-speed ferries (≥30 knots) (Aguilar et al., 2000; de Stephanis and Urquiola, 2006; Ritter, 2010). Vanderlaan and Taggart (2007), used North Atlantic right whale ship strike data to develop a model of the probability of mortality based on strikes occurring at different speeds, regardless of vessel size. The authors suggested that strikes at speeds over 18 knots were fatal almost 100% of the time.

When whales get hit by a vessel, they can present: sharp trauma lesions, generated by the propeller or the keel of the vessel, and/or, blunt trauma lesions, caused by a non-rotating feature of the vessel, like the hull or the skeg (Campbell-Malone et al., 2008; Moore et al., 2013). Injuries within the first category, usually involve the presence of one or more linear to curvilinear laminar incising wounds, that usually cause damage to the underlying soft tissue. Extreme injuries, frequently lethal, involve damage to the axial musculature or the vertebral column affecting locomotion, or even the complete separation of part of the body, with severe central nervous system (CNS) injury. In blunt traumas, areas of hemorrhage and edema in the blubber, subcutaneous tissue, and skeletal muscle are common features, as well as luxations and/or fractures, usually concomitant. In more severe cases, rupture of internal organs can be observed (Campbell-Malone et al., 2008; Moore et al., 2013). Full necropsies should be carried out as some injuries, especially those related with blunt trauma, may not be apparent externally. In very decomposed carcasses, differentiation between ante-mortem lesions and post-mortem changes can be very challenging (Campbell-Malone et al., 2008; Moore et al., 2013).

Fat embolism is defined as the mechanical obstruction of blood vessels by circulating fat particles (Watson, 1970; Hulman, 1995). In humans it is usually related with traumas involving long and pelvic bones (Watson, 1970; Fulde and Harrison, 1991). After trauma, fat cells from the bone marrow of fractured bones or from damaged soft tissues, enter the bloodstream through torn venules in the injury or fracture site, and typically first arrive to the lungs where they may get trapped within the pulmonary microvasculature (<20 μm in diameter) (Watson, 1970). For this reason, the lung is considered a target organ for fat emboli detection (Levy, 1990). The presence of fat emboli within the lungs constitutes evidence of *antemortem* injury, as cardiac function is needed, even for a short time, to allow the circulation of fat droplets to the lungs (Armstrong et al., 1955; Mason, 1968; Saukko and Knight, 2004). It is a common and usually asymptomatic finding (Watson, 1970; Fulde and Harrison, 1991), that infrequently leads to a clinical disorder known as fat embolism syndrome (Glover and Worthley, 1999). Its severity has been related to the multiplicity of the fractures, and it occurs very rapidly after severe trauma (Tanner et al., 1990), being also present in those cases in which the death occurs immediately after the trauma (Emson, 1958).

In the Canary Islands, over 57% of the sperm whales stranded since 2000 presented evidence of ship strike, and over 70% of them were in an advanced or very advanced decomposition state (Arbelo et al., 2013; Díaz-Delgado et al., 2018). Thus, we aimed to analyze lungs from sperm whales dead in Canarian waters between 2000 and 2017 with signs of ship strike to determine: (1) if fat embolism is a common finding in sperm whale's lung tissue, (2) if the presence of fat emboli within the lung vessels is a useful diagnostic tool to assess ante-mortem ship strikes, and (3) if lung fat emboli density relates to the severity or location of the traumatic injuries.

MATERIALS AND METHODS

Animals Included in the Study

Between January 2000 and December 2017, 35 sperm whales encountered dead, floating or stranded, in the Canary Islands (28°N, 16°W; Spain) were necropsied, following standardized protocols (Kuiken and García Hartmann, 1991), to find out the cause of death. 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.

Age categories were established based on total body length (Perrin et al., 2009) and histologic gonadal examination, including: neonate, calf, juvenile, subadult and adult (Geraci and Lounsbury, 2005). Decomposition code was established according to Kuiken and García Hartmann (1991) classification, with a modification for code 1: code 1 for "very fresh" was assigned to an animal that has recently died. The other codes remained the same: code 2 for "fresh dead animals" (no bloating nor changes in coloration, eatable meat), code 3 for "moderate autolysis" (may present with some skin desquamation, the carcass might have started to swollen, and organs may have changed coloration and more friable), code 4 for "advanced autolysis" (skin desquamation, swollen carcass, organs difficult to recognize), and code 5 for "very advanced autolysis" (the skin may be absent, some or all organs may be liquefied, also mummification or adipocera may be observed in some carcasses). Body condition was determined based on anatomical parameters such as the presence of certain prominent bones, the dorso-axial muscular mass, and the presence or absence of fat deposits (Joblon et al., 2014).

The lungs of 16 sperm whales with evidence of ship strike and eight sperm whales without (control group), were studied to detect fat emboli (**Table 1**) using the osmium tetroxide (OsO_4) technique.

Osmium Tetroxide Technique

A retrospective study was carried out using lung tissue samples fixed in 10% buffered formalin between 2000 and 2017 and kept in the Institute of Animal Health Tissue Bank. Formalin-fixed lung samples were cut into thin sections ($\leq$1 mm) to ensure the proper penetration of OsO_4. Post-fixation with OsO_4 is needed as lipids are soluble in the processing solvents used to embed the tissues in paraffin. The sections were then washed with running tap water for 20 min followed by 10 min in distilled water. Next, the sections were immersed in 1% OsO_4 aqueous solution (sonication was previously used to dissolve the commercial crystalline OsO_4) within hermetically sealed bottles on a shaker inside a chemical hood. Then, the sections were rinsed in running tap water for 30 min and immersed in 1% periodic acid until the dark osmicated tissues were uniformly cleared (Abramowsky et al., 1981). Samples were washed for 30 min with tap water, and rinsed three times with distilled water. Then, the samples were routinely processed and embedded in paraffin-wax, sectioned at 5 μm-thick, treated with picric acid (1% in ethanol 96%) for 24 h to remove excess formalin pigment (Abramowsky et al., 1981), and counterstained with hematoxylin and eosin (HE). Finally, slides were mounted in DPX mounting medium. Tissue sections as blubber and *rete mirabile* (which have abundant adipocytes), were used as positive controls (treated with OsO_4) and negative controls (non-treated with OsO_4) to validate the technique.

Microscopic Analysis

All lung sections were evaluated for the presence/absence of fat emboli, as well as the area occupied by those emboli within lung vessels using light microscopy (Olympus BX51).

Each lung tissue section was divided in "N" number of 100× magnified microscopic fields (MF) (Ocular: 10× and Objective: 10×). A Bootstrap analysis was carried out to determine the number of 100 MFs ("n") that needed to be studied for each tissue section (**Table 2**). We considered a good estimation of the true value if the total bound error was below 8%. Depending on the total tissue section area the Bootstrap analysis yielded results between 14 and 20 100 MFs. These fields were randomly selected and photographed using an Olympus XC30 camera (Olympus Soft Imaging Solutions GmbH©, Johann-Krane-Weg 39, D-48149 Münster) (**Table 2**). Fields containing pleura (adipocytes are normally present in the pleura of sperm whales) (**Figure 1B**), and/or large-diameter bronchi/bronchioles (empty spaces) were discarded to ensure a similar parenchyma size comparison between the different fields.

The software ImageJ (1.48v, Wayne Rasband, National Institute of Health, United States) was used to determine the area occupied by fat emboli, in pixels, in each of the photographs. Each of the 100 MFs' photographs has a total area of 1,920,000 pixels. Fat emboli are recognized as black droplets primarily in the lumen of capillaries and small- and medium-size arteries. The software ImageJ allows the quantification of areas of a certain color automatically, or the quantification of selected areas manually. We manually selected the fat emboli areas in the lung parenchyma, as not all black areas were fat emboli [e.g., various artifacts, fat in bronchioli and/or alveoli (**Figure 1C**)], and the emboli were not homogenously stained. As a result, for each animal, we ended up having an "n" number of 100 MFs (photographs), each of them with an area in pixels occupied by fat emboli.

Analysis for the Validation of the Osmium Tetroxide Technique as a Complementary Diagnostic Tool for Ship-Strikes

The 25th, 50th, 75th, and 90th percentiles of the areas (pixels) occupied by fat emboli in the "n" 100 MFs studied were calculated for each animal (**Table 3**). As fat emboli were also present in a few lungs of the "control group," a classification and regression tree (CART) was developed to obtain a cut-off value from which the probability of association with ship-strike is high. This procedure classifies data using a sequence of if–then rules. The basis of the decision tree algorithms is the binary recursive partitioning of the data. The most discriminative variable is first selected to partition the data set into child nodes. The splitting continues until some stopping criterion is reached. The tree was constructed according to the following algorithm: in the first stage, the tree grows until all cases are correctly classified, and in the second stage, we used the tenfold cross-validation method of successive pruning (Breiman et al., 1984). Finally, the tree that minimized the error measurement (deviance) was chosen. Then, the low and high-probability categories obtained were compared using the exact Fisher test. The sensitivity

TABLE 1 | Epidemiological and biological data of the sperm whales included in the present study with evidence of ship strike.

Case	Age	Sex	Strand. locat.	Strand. date (dd/mm/yy)	Length (cm)	Decomp. code	Body condition	Food content	Type trauma	Trauma location	Fractured bones
Ship strike group											
(1)	J	F	NE-T	21/08/01	600	4	4	Fresh	Sharp	Abdomen	0
(2)	C	M	SE-T	05/07/03	530	3	4	Digested	Both	Head	0
(3)	S	F	S-F	11/05/05	750	4	4	Digested	Sharp	Abdomen	0
(4)	C	F	SE-T	27/04/06	460	2	3	Milk	Sharp	Thorax and Abdomen	Ribs
(5)	C	F	SE-GC	10/04/08	500	4	NE	NE	Sharp	Thorax	Ribs
(6)	A	F	SE-T	13/03/09	915	2	3	Digested	Sharp	Abdomen	NE
(7)	C	M	E-T	13/03/09	570	3	4	Digested	Sharp	Abdomen	Vertebrae
(8)	J	M	NE-T	09/07/10	785	4	4	Semi-digested	Sharp	Head	Skull
(9)	C	M	SE-GC	29/09/11	550	4	NE	Digested	Sharp	Thorax	Ribs
(10)	C	M	N-GC	26/04/11	575	4	2	Digested	Both	Thorax and Caudal third	0
(11)	J	M	NE-H	09/02/12	NE	5	NE	NE	Both	Head and Caudal Third	Mandible and Caudal Section
(12)	C	M	NE-F	24/04/12	552	4	4	Digested	Sharp	Neck	0
(13)	C	F	NW-L	21/06/12	550	5	NE	Semi-digested	Both	Head and Caudal Third	Mandible and Caudal Section
(14)	C	F	S-T	19/10/12	365	4	NE	NE	Sharp	Abdomen	Vertebrae
(15)	C	M	NE-GC	09/05/13	435	4	3	None	Sharp	Head	Skull
(16)	A	F	NE-T	05/06/14	825	4	3	Fresh	Sharp	Abdomen	Vertebrae
Non-ship strike group											
(17)	N	F	W-LG	22/07/08	340	1	NE	None	None	None	0
(18)	A	F	SW-T	27/06/09	940	3	2	None	None	None	0
(19)	A	F	N-T	08/09/11	1050	4	2	NE	None	None	0
(20)	A	F	NE-T	15/03/13	950	3	3	Digested	None	None	0
(21)	J	F	N-GC	29/05/13	790	4	3	Digested	Blunt	Head and Thorax	0
(22)	A	F	N-L	17/03/17	900	4	NE	Digested	None	None	0
(23)	S	F	S-H	08/10/17	905	4	3	Digested	None	None	0
(24)	J	F	E-GC	25/10/17	840	4	NE	Digested	Blunt	Caudal Third	0

Age: N, Neonate; C, Calf; J, Juvenile; S, Subadult; A, Adult. Stranding location: N, North; S, South; E, East; W, West; NE, Northeast; NW, Northwest; SE, Southeast; SW, Southwest; T, Tenerife; GC, Gran Canaria; F, Fuerteventura; L, Lanzarote; H, El Hierro; LG, La Gomera. Decomposition code: 1, Very fresh; 2, Fresh; 3, Moderate autolysis; 4, Advanced autolysis; 5, Very advanced autolysis. Body condition: 1, Very poor; 2, Poor; 3, Fair; 4, Good; NE, Not Evaluated.

TABLE 2 | Number of microphotographs captured based on the total number of 100 magnifications microscopic fields (100 MFs) of the tissue section.

N	≤20	20–30	31–40	41–50	51–60	61–70	≥70
n	14	15	16	17	18	19	20

"N" is the total number of 100 MFs analyzed in the lung tissue section; and "n" is the number of microphotographs that should be captured to achieve <8% error bound.

and specificity were estimated by means of 95% confidence intervals (95% CI).

Exploration of Association Between Trauma-Related Variables and Fat Emboli Severity

Categorical variables were expressed as frequencies and percentages, and continuous variables, like fat emboli areas, were expressed as medians and interquartile ranges (IQR = 25th–75th percentile) (**Table 3**).

The variables age, presence/absence stomach food content and degree of digestion of the ingesta, presence of fractures and stranding location were compared between both groups using the Chi-square ($\chi 2$) test or the exact Fisher test for percentages; and the Wilcoxon test for independent data for the medians (**Table 3**).

The variables age, sex, body condition, presence/absence and degree of digestion of the stomach food content, trauma location

FIGURE 1 | Lung sections treated with OsO_4. **(A)** Appearance of a lung parenchyma negative to fat emboli (Bar = 500 μm). **(B)** Black-stained adipocytes in the pleura (arrows) (Bar = 200 μm). **(C)** Black-stained fat droplets within a bronchiole (arrows) (Bar = 200 μm).

and presence/absence of fractures were analyzed within the group with evidence of ship strike to assess potential associations with fat emboli severity (lung area occupied by fat emboli) (**Table 1**). For this aim, a linear analysis was carried out. The variables introduced in the model were age (calf/not calf), sex, body condition, presence/absence and degree of digestion of the stomach food content, trauma location and presence/absence of fractures. Then, a selection of variables based on the Akaike information criteria was performed.

Data were analyzed using the R package, version 3.3.1 (R Development Core Team, 2016).

RESULTS

Presence of Fat Embolism

A total of 83% (13/16) sperm whales with evidence of ship strike (**Figure 2**) had intravascular OsO_4-positive fat emboli. Fat emboli ranged from 67 to 59773 pixels, and were seen circulating in medium and small caliber intrapulmonary arteries and/or obliterating arterioles and capillaries, both in fresh and decomposed specimens (**Figure 3**). None but two of eight "control" sperm whales had detectable fat emboli (**Figure 1A**). Those two animals (cases 21 and 24) had rare isolated OsO_4-positive fat emboli (<650 pixels) in arterioles (**Table 3**).

Calves were "significantly" (p = 0.003) more likely to be involved in ship-strikes than other age categories. As well, the presence of fractures was "significantly" associated to ship-strikes p = 0.003. Other variables studied like the stranding location (island) or the presence/absence and degree of digestion of the ingesta, were not significantly different between both groups (**Table 3**). An association between trauma-related variables and fat emboli severity could not be established.

When assessing the probability of ship strike based on the fat emboli area, significant differences between non-strike and strike groups started to be seen in the 50th-percentile values, but the

TABLE 3 | Categorical variables studied expressed as frequencies and percentages or continuous, expressed as medians and interquartile ranges (IQR = 25th–75th percentile).

	Ship strike		
	No *N* = 8	**Yes *N* = 16**	***P***
Length (cm)	902 (828–942)	552 (515–675)	0.010
Calf	0	10 (66.7)	0.003
Fracture	0	10 (66.7)	0.003
Location			0.661
Tenerife	3 (37.5)	8 (50.0)	
Gran Canaria	2 (25.0)	4 (25.0)	
El Hierro	1 (12.5)	1 (6.2)	
Fuerteventura	0	2 (12.5)	
Lanzarote	1 (12.5)	1 (6.2)	
La Gomera	1 (12.5)	0	
Food			0.470
No content	2 (25.0)	1 (6.2)	
Milk	0	1 (6.2)	
Fresh	0	2 (12.5)	
Semi-digested	0	3 (18.8)	
Digested	5 (62.5)	6 (37.5)	
Not determined	1 (12.5)	3 (18.8)	
Fat emboli area			
Percentile 25	0 (0–0)	0 (0–1064)	0.085
Percentile 50	0 (0–0)	0 (0–1748)	0.033
Percentile 75	0 (0–0)	830 (0–3249)	0.003
Percentile 90	0 (0–8.38)	5274 (132–10044)	0.006
Probability of ship strike			<0.001
Low: No calf and Fat 0.75 ≤ 140	8	1	
High: Calf or Fat 0.75 > 140	0	15	

In the last column, P-value <0.05 was considered as statistically significant difference between ship-strike and non-ship strike groups.

FIGURE 2 | Vessel strike-related injuries in sperm whales (*Physeter microcephalus*) stranded in the Canary Islands. **(A)** Presence of a deep incision in the right flank of the animal, caudal to the pectoral fin, with soft tissue exposure, abdominal perforation and evisceration and costal fractures, case 4. **(B)** Complete amputation of the vertebral column at the level of the last thoracic vertebrae, case 14.

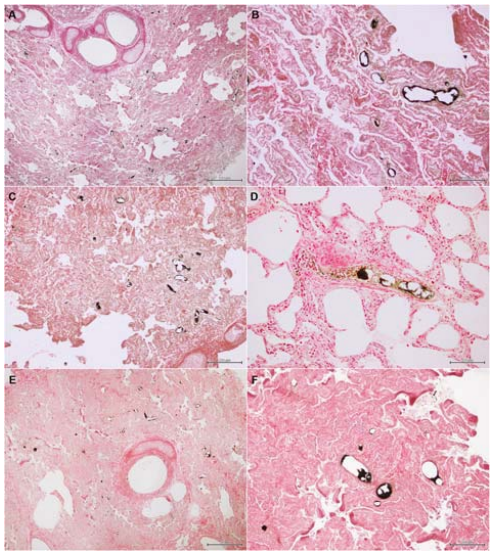

FIGURE 3 | Black stained (OsO_4-positive) fat emboli in the lung microvasculature, mainly arterioles, of some of the sperm whales stranded in the Canary Islands with signs of ship strike **(A–F)**. Bar: A = 500 μm; B = 100 μm; C = 200 μm; D = 100 μm; E = 500 μm; F = 200 μm.

highest discriminant power between both groups corresponded to the fat emboli area's value of the 75th-percentile (**Table 3**). The CART indicates that if the value of the 75th-percentile fat emboli area is greater than 140 pixels in the animal studied (cut-off value), the probability of having been hit by a vessel is high, and so the animal is assigned to the strike group. If the value of the area occupied by fat emboli in the 75th-percentile is lower than 140 pixels, and the animal is a calf, it is also assigned to the strike group. If none of the previous conditions are met, the animals are assigned to the non-strike group ($p < 0.001$) (**Table 3** and **Figure 4**). The sensitivity and the specifity were 89% (52–100; 95% CI) and 100% (78–100; 95% CI), respectively.

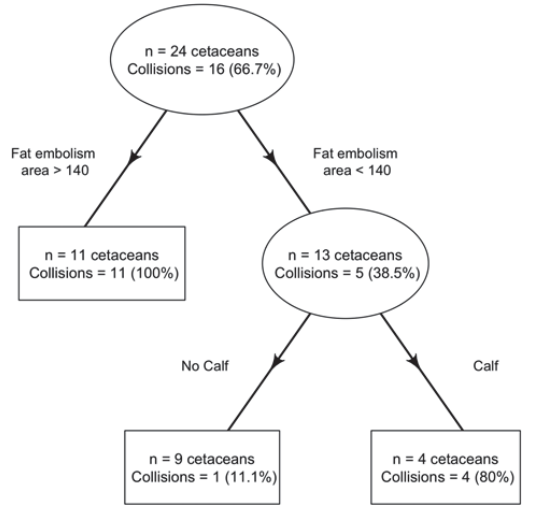

FIGURE 4 | Result of the classification and regression tree to determine the cut off for ship-strike probability of the studied sperm whales. A high or low probability of ship strike was established according to: first, the fat emboli-P75 value and secondly, being a calf or not a calf. If fat emboli area <140 and Not a Calf → Non-strike group; If fat embolism area <140 and Calf → Strike group; and if fat embolism area >140 → Strike group. Its sensitivity is 89% (95% CI = 52–100%) and its specificity 100% (95% CI = 78–100%).

Factors Related to Ship Strikes in the Canary Islands

Most sperm whales with evidence of ship-strike were calves (10/16; 62.5%), followed by juveniles (3/16; 18.75%), adults (2/16; 12.5%) and subadults (1/16; 6.25%). All the adults/subadults included in this study were females.

Most of the animals with evidence of ship strike appeared floating or stranded along the east coast of Tenerife (56.25%), followed by the east coast of Gran Canaria (18.75%), east coast of Fuerteventura (12.5%), and finally east coast of El Hierro (6.25%) and west coast of Lanzarote (6.25%) (**Figure 5**).

DISCUSSION

Presence of Fat Embolism and Its Significance in Ship Strikes

The lack of detectable fat emboli in lung tissue of most control sperm whales suggests that fat embolism is not a physiological or common finding in lungs of stranded sperm whales. In addition, the presence of abundant OsO_4-positive fat emboli in most sperm whales with evidence of ship-strike indicates an association with trauma. The etiology of fat emboli in the blood stream may be trauma- or non-trauma related (Glover and Worthley, 1999). Trauma conditions may include marrow-containing bone fractures or adipocyte-rich soft tissue injuries (Watson, 1970; Fulde and Harrison, 1991;

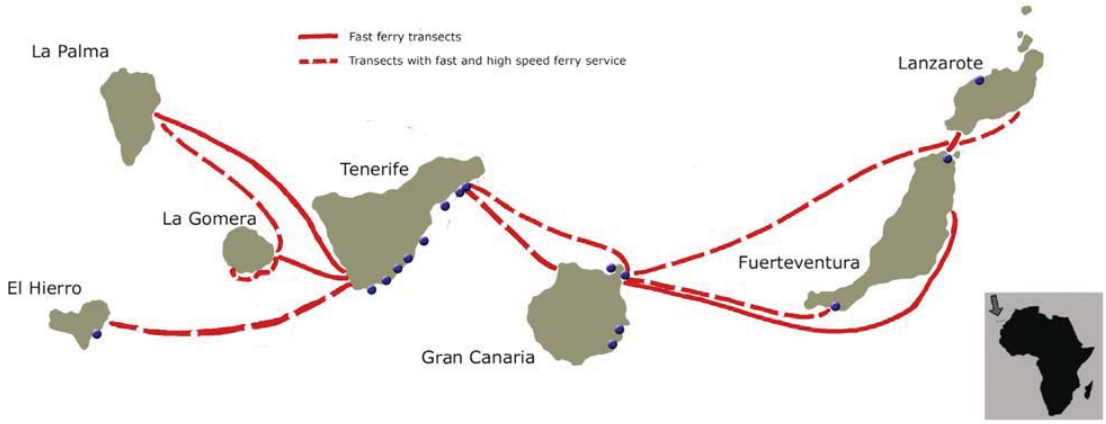

FIGURE 5 | Inter-island ferry transects in the Canary Islands (different speed-ferries are represented with different line types), and stranding locations (blue dots) of the sperm whales with evidence of ship strikes in the Canary Islands [modified from Ritter (2010)].

Gupta and Reilly, 2007). Both possibilities likely coexisted in our cases with evidence of ship-strike. The presence of fat emboli in the lung microvasculature indicates that the animal was alive at the moment of the strike and that cardiovascular collapse did not ensue immediately with successful pulmonary irrigation for an unknown period of time.

In addition to fat emboli, other typical findings of *antemortem* ship strike in cetaceans include subcutaneous, muscular and/or internal hemorrhage with hematoma formation, organ contusion and/or rupture with bleeding, e.g., in airways, in gastrointestinal tract, and edema in various organs mainly due to increased hydrostatic pressures, increased permeability due to hypoxia and vascular disruption (Campbell-Malone et al., 2008; Moore et al., 2013). Traumatic injuries on the dorsum are usually considered as *antemortem* or *perimortem* as carcasses tend to float with the ventral or lateral side upward, making a dorsal strike of a carcass unlikely (Laist et al., 2001; Campbell-Malone et al., 2008). On histopathologic examination, the presence of inflammatory response, hemorrhage or edema (Campbell-Malone et al., 2008; Moore et al., 2013), as well as acute, monophasic myocyte (segmentary, discoid) degeneration, contraction band necrosis, and/or fragmentation of the myofibers in the skeletal muscle (Sierra et al., 2014) support *antemortem* trauma.

Nevertheless, when working with decomposed carcasses it is not always feasible to assess many of the ship strike evidences described above. It is in these cases, where the detection of fat emboli in the lungs has proven to be a valuable and reliable confirmatory diagnostic tool, allowing us to conclude that, at least, 83% of the studied stranded sperm whales in the Canary Islands with evidence of ship strike were alive at the moment of the strike.

All the same, the methodology for fat emboli detection is not devoid of certain limitations that may lead to an underestimation of fatal vessel strikes, such as the slanted and arbitrary sampling of lung tissue. Kinra and Kudesia (2004), suggested that fat emboli are not homogenously distributed along lung tissue. Although different lung areas, including cranial, medial, and caudal samples from both lungs should be routinely collected during the necropsy, there is not a specific sampling protocol to accurately assess lung fat emboli. Thus, results from small lung portions taken arbitrarily should be carefully interpreted as they may not be representative of the whole tissue. Future anatomical and topographical studies of pulmonary blood circulation and fat emboli distribution, respectively, are necessary to assess which lung areas should be sampled for an accurate fat emboli detection.

Microscopically, the severity of fat embolism has been traditionally assessed using a simple scale based on the number of emboli encountered in the tissue section studied (Saukko and Knight, 2004). Here we proposed and evaluated fat emboli area as a better estimator to assess fat emboli severity since the same number of emboli in two different lung histological sections may occupy different areas, and the one with the largest area occupied, would be a more severe case.

The rule developed for our samples based on the area occupied by fat emboli allowed us to discriminate between sperm whales that suffered strikes from those that died due to other causes, even when fat emboli was present in some animals of the control group (Cases 21 and 24). In these two cases, there was a blunt trauma of unknown origin. Possible etiologies included intra- or interspecific interactions or a potential live-stranding event (Díaz-Delgado et al., 2018). Intra-/interspecific traumatic interactions are frequent among cetaceans, and may result in blunt traumas where internal hemorrhages and/or bone fractures may occur (Arbelo et al., 2013; Díaz-Delgado

et al., 2018). In the case of sperm whales, they have been observed being attacked by killer whales (*Orcinus orca*) or male sperm whales fighting with each other (Whitehead, 2009). These interactions have been observed to occasionally cause fat emboli (Díaz-Delgado et al., 2018).

When relating the severity of fat embolism and the severity of the trauma a general positive correlation was established by Emson (1958) based on the type and number of bones fractured of 100 patients who died after injury. On the contrary, we did not find any association between variables related to trauma (i.e., presence of fractures or location of the trauma) with fat embolism severity. This could be due to the low sample size, heterogeneous distribution of fat emboli, methodological bias, immediate cardiovascular collapse with none or little pulmonary irrigation post-trauma (sudden death), or to the fact that the strike occurred *post-mortem*. Fatal lesions, often involving direct cardiovascular trauma with or without rupture of large vessels (hypovolemic shock) and/or severe neurogenic dysfunction, may cause an abrupt death, leading to immediate cease of the blood, and could explain the absence of fat emboli in different body organs, including the lung. This has been documented in aircraft fatalities, were extensive injuries were associated with lower grades of fat embolism (Mason, 1962), or no emboli at all in disintegration cases (Kinra and Kudesia, 2004), showing the importance of intact circulation for the formation of fat emboli. We surmise rapid cardiovascular collapse could explain lack of detectable fat emboli in three of our cases (cases 7, 10, and 13). These animals presented severe injuries, including abdominal evisceration or caudal amputation (Díaz-Delgado et al., 2018).

Factors Related to Ship Strikes in the Canary Islands

All the sperm whales included in this study, were either female adults/subadults, or juveniles or calves, of any sex. This is in agreement with the fact that sperm whale females and their progeny composed the main groups present all year round in Canarian waters, which are considered nursery and breeding areas (André, 1997).

The fact that young animals are not fully adapted to dive and need to spend more time at surface, together with their relatively slow swimming speed compared to adults, may explain their higher vulnerability to ship strikes (Papastavrou et al., 1989; Laist et al., 2001; Whitehead, 2009). Mothers with recent calves (cases 6 and 7) may be also at higher risk as they will spend more time in the surface with their offspring.

A previous study estimated the absolute abundance and density of sperm whales in Canarian waters, and concluded that the species would not be able to sustain the current level of strikes (Fais et al., 2016). This impact is aggravated by the female philopatry in the Canaries, as they are not genetically connected to west North Atlantic populations (Alexander et al., 2016), and by the number of calves and reproductive females affected by strikes.

Most struck sperm whales appeared in the east coast of Tenerife, in agreement with previous studies (André, 1997; Carrillo and Ritter, 2010; Ritter, 2010). A major explanation for this is that the channel between Tenerife and Gran Canaria is a prime habitat for sperm whales in the Canaries (André, 1997; Fais et al., 2016), as well as an area with a high maritime traffic density, dominated by fast and high speed ferries (Ritter, 2010). An overlap between most of the sperm whales' stranding locations and fast-ferry transects was also observed in the present study.

To conclude, this study provided compelling histochemical evidence of fat emboli as a reliable confirmatory diagnostic tool of ante-mortem ship-strike even in decomposed sperm whale carcasses. Our results demonstrated that most of the sperm whales with evidence of ship-strike and stranded in the Canaries were alive at the moment of the strike. However, this may be an underestimation as cases where immediate cardiovascular collapse (sudden death) occur, may lack detectable fat emboli in the lungs. A final diagnosis of antemortem ship-strike may considerably benefit from fat emboli detection in lung tissue, particularly when other trauma-related gross and microscopic findings are not evident.

Some future directions may include the study of fat emboli distribution within the lungs to determine if some areas are more affected by fat emboli, and based on the results, the development of a homogenized lung sampling protocol to detect fat emboli. Alternative techniques to osmium tetroxide, which is extremely toxic, should be developed to study lipids histologically. Lipid composition analyses of fat emboli may contribute to a better understanding of its pathogenesis in these animals.

Some mitigation measures have been implemented in other locations and have proven to be effective, such as a mandatory vessel-speed restriction in the United States East Coast (Conn and Silber, 2013), the establishment of Traffic Separation Schemes (TSS) in the Bay of Fundy (Vanderlaan et al., 2008) or the proposal of recommended Areas To Be Avoided (ATBA) like the Roseway Basin Area (Vanderlaan and Taggart, 2009). Similarly, mitigation measures to reduce ship-strike mortalities and guarantee the survival of the sperm whales' population in Canarian waters should be further explored and implemented.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

ETHICS STATEMENT

All animals included in the present study were dead, either floating offshore or stranded in the coast. 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

AF: conceptualization. MArb, ES, YBdQ, JD-D, CS-S, RP-L, and MArr: sampling. MArr: laboratory analyses. PS, MArr, and YBdQ: data analyses. AF: funding. MArr: writing. All authors: review and editing. YBdQ and AF: supervision.

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Comparison of Three Histological Techniques for Fat Emboli Detection in Lung Cetacean's Tissue

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Fat embolism is the mechanical blockage of blood vessels by circulating fat particles. It is frequently related to traumas involving soft tissues and fat-containing bones. Different techniques have been used for decades to demonstrate histologically fat emboli, being the extremely toxic post-fixation with osmium tetroxide one of the most used techniques in the last decades. In the present study, the osmium tetroxide technique was compared qualitatively and quantitatively, for the first time, with chromic acid and Oil Red O frozen techniques for histological fat emboli detection in the lungs of eight sperm whales that died due to ship strikes. This was also the first time that chromic acid technique was tested in cetaceans. Results showed that the three techniques were valuable for the histological detection of fat embolism in cetaceans, even when tissues presented advanced autolysis and had been stored in formaldehyde for years. Although quantitative differences could not be established, the Oil Red O frozen technique showed the lowest quality for fat emboli staining. On the contrary, the chromic acid technique was proven to be a good alternative to osmium tetroxide due to its slightly lower toxicity, its equivalent or even superior capacity of fat emboli detection, and its significantly lower economic cost.

Fat embolism is defined as the mechanical obstruction of blood vessels by circulating fat particles[1,2]. The lung has traditionally been considered the target organ for fat emboli detection, as the pulmonary microvasculature (<20 µm in diameter) blocks the circulation of the fat droplets, acting as a filter to protect the systemic circulation[2,3].

Different techniques have been used for histological fat emboli demonstration, usually under light microscopy or less frequently using electron[4,5] or most recently, confocal microscopy[6,7]. While the electron and confocal microscopy techniques are very interesting and promising technologies for the investigation on different aspects of fat emboli, they require expensive equipment and trained personnel thus, they are not accessible to many diagnostic laboratories. At the same time, the more classic histopathological techniques are routinely done by most laboratories and can provide a proper diagnosis. Considering this, the present study will be focused on histological fat embolism diagnosis using light microscopy.

Among the various techniques used for lipid demonstration in frozen sections of fresh or formalin-fixed tissues, the solvent dyes Sudan III and IV[8,9] were the first to be used (e.g.[10–12]). Later, other solvent dyes as Sudan Black B or Oil Red O, argued to give superior results[13,14], started to be commonly used[1,15–17]. Frozen techniques for lipid staining comprise lower health risks but have been argued to give lower morphology quality and to show less capacity to detect small quantities of lipids in comparison to techniques in paraffin-embedded tissues[18,19].

In paraffin-embedded tissues, osmium tetroxide (OsO_4) has been for decades the technique of choice for histological demonstration of fat embolism due to its high quality[20–24]. As lipids are soluble in the processing solvents used to embed the tissues in paraffin, formalin-fixed tissues should be treated with OsO_4 to fix the lipids to the sample before paraffin embedding. Nevertheless, when working with OsO_4 safety measures should be strictly followed as it is known to be extremely toxic. Its vapors can lead to severe eye irritation, including cornea damage, and it is listed by the Globally Harmonized System of Classification and Labeling of Chemicals (GHS)[25] as fatal if inhaled, swallowed or in contact with skin[26]. In the last decades, Tracy & Walia (2002) have described

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another post-fixation technique that uses chromic acid to fix lipids by transforming fat emboli in organometallic complexes insoluble in organic solvents and stainable in paraffin sections[4]. Some advantages of chromic acid when compared with OsO_4 may include its lower price, higher capacity of tissue penetration, and its lower degree in toxicity in some GHS hazard statements. However, chromic acid is a very hazardous and possibly carcinogenic chemical that, as OsO_4, should be handled carefully and safely[27].

Fat embolism has been extensively described, both in human and animal science, associated with a variety of traumatic and non-traumatic processes[28,29], comprising the majority of the cases traumas to soft tissues and fat-containing bones, especially long bones and pelvis[2,30]. Moreover, the histologic detection of lung fat emboli has been used for decades in forensic practice to demonstrate *antemortem* violence, as cardiac function is needed, even for a short time before death, to allow the circulation of fat emboli to the lungs[10,31–33].

The diagnosis of fat embolism is based on the histological demonstration of fat emboli within vascular vessels. However, not only their presence or absence within the tissue section should be assessed but also graded on a scale. This is necessary because the higher the severity, the greater the probability that fat embolism is involved in the patient´s death. Although there is not a universally accepted system to grade fat embolism, previous studies in the literature have traditionally graded it using semi-quantitative indexes, which are based on the number of emboli encountered in the tissue section analyzed, like Falzi, Sevitt or Mason's scales[31,34,35]. Some limitations of these semi-quantitative grading systems include that they are based on the observer's personal criteria, that they do not take into account the size of the emboli (which is expected to play a role in severity), and finally, that they do not consider the total tissue section area, which has been mentioned by previous authors as important to accurately assess the degree of fat embolization[6,36].

In addition to the need of using objective quantitative scales rather than semi-quantitative indexes to properly assess the morphological severity of fat embolism, it is important to determine if the most commonly used techniques for fat embolism demonstration are equally efficient in the detection and quantification of those emboli, with the objective of using the less toxic but efficient technique. However, to the best of our knowledge there is only one study in the literature that has compared two techniques, the OsO_4 and Oil Red O frozen section techniques, for their capacity to detect fat emboli but did it in an eye-based study rather than a proper quantitative comparison[18].

Contrary to the high number of studies in literature addressing fat embolism in different species, especially humans, studies on fat embolism in cetaceans are extremely scarce. This is explained because the field of pathology and histopathology in these animals constitutes a recent research field. Moreover, laboratory techniques and protocols need to be adequate for these species' tissues.

In cetaceans, fat embolism has been described due to intra-interspecific traumatic interactions[37], in combination with gas embolism (gas and fat embolic syndrome) in beaked whales that stranded in temporal and spatial association with naval military manouvres[38,39], and in animals that have been struck by vessels in the Canary Islands[37,40–42]. In this last case of ship strikes, lung fat emboli have proven to be a confirmatory diagnostic tool of *antemortem* strikes, especially in decomposed carcasses in which the assessment of tissular changes characteristic of an *antemortem* trauma can be challenging. In relation to this, the study carried out by Arregui *et al*. in 2019, focused on sperm whales and ships, demonstrated that at least 80% of the specimens with evidence of ship-strike in this area were alive at the moment of the strike and that mitigation measures need to be implemented[42].

Considering all this, the objectives of the present study were first, to evaluate the capacity of chromic acid to demonstrate fat emboli in cetacean tissues; and secondly, to compare qualitatively (e.g. intensity of the staining or the precise location of fat emboli within vessels) and quantitatively (to assess if they detect a similar quantity of fat emboli) the capacity of fat emboli detection between OsO_4 to both: the Oil Red O frozen and the chromic acid techniques.

To the best of our knowledge, this is the first study evaluating the chromic acid technique in cetacean lungs positive to fat embolism and the first time a detailed quantitative and qualitative comparison is made among the three techniques for fat emboli detection.

Material and Methods

Animals included in the study. To assess and compare the ability of the three histochemistry techniques (i.e.: chromic acid and OsO_4 in paraffin tissues and Oil Red O in frozen tissues) to stain fat emboli, we selected eight sperm whales, in different conservation codes, which had signs of ship strikes and were OsO_4-positive to lung fat emboli with different degree of severity[42].

Lung samples were collected following standardized necropsies[43]. For decomposition status, five codes were applied[43]: code 1 for "very fresh", was assigned to an animal that has recently died, code 2 for "fresh dead animals" (no bloating nor changes in coloration, eatable meat), code 3 for "moderate autolysis" (may present some skin desquamation, the carcass might have started to swollen, and organs may have changed coloration and be more friable), code 4 for "advanced autolysis" (skin desquamation, swollen carcass, organs difficult to recognize), and code 5 for "very advanced autolysis" (the skin may be absent, some or all organs may be liquefied, and mummification or adipocere may be observed in some carcasses). Among the eight animals included, there was one fresh animal (code 2), one moderately decomposed animal (code 3), five advanced-autolytic animals (code 4), and one very advanced-autolytic animal (code 5). The bias of the study towards decomposed animals was explained because most of the sperm whales that appear floating or stranded in Canarian coasts presented and advanced decomposition state[37,40].

Description of the techniques used for fat emboli detection. After collection, lung samples were immediately immersed in 10% neutral buffered formalin for fixation and stored in the Institute of Animal Health Tissue Bank until being processed for the present study. They were treated for fat emboli detection following three techniques, two of them in paraffin-embedded tissues ("OsO_4 and chromic acid techniques") and the third one using frozen sections ("frozen section technique").

As lipids are soluble in the solvents used to embed the tissues in paraffin, OsO_4 or chromic acid were used in two of the techniques to fix the lipids to the tissue prior to paraffin embedding. In the frozen section technique, lipid fixation was not needed as tissues were not embedded in paraffin.

To compare the OsO_4 technique with both, the chromic acid- and the frozen section technique, formalin-fixed lung sections were cut in half, being one of the faces treated following the chromic acid technique, and the other one with the OsO_4 technique. Then, the same or a different lung area was cut in half and one of the faces was treated with OsO_4 and the other with the frozen section technique, in order to compare both.

Sections of blubber, a specialized hypodermis, and *rete mirabile*, a complex structure of vessels, both presenting an abundant number of adipocytes, were used as positive controls, when treated with the different techniques; and as negative controls, when non-treated by the different techniques. Sperm whales´ lungs negative to fat emboli were also used as negative controls.

All the work in the laboratory was performed following strictly the handling and protection measures detailed in the different reagents' safety data sheets.

OsO_4 technique. OsO_4 crystals from one-gram commercial ampoule were carefully introduced in distilled water. Distilled water was pipetted several times inside the ampoule to take out all the crystals. The solution was then sonicated in periods of 10 minutes with 2 hours break until all the crystals dissolved. When sonicating, ice brick block freezers were placed in contact with the bottle containing the solution to prevent the solution from heating. During the breaks, the solution was kept hermetically sealed in the fridge at 4 °C. Afterwards, the protocol developed by Abramowsky and colleagues (1981) was followed to fix/stain with OsO_4 to demonstrate fat in paraffin sections[18,42]. Hematoxylin and eosin were used for counterstaining.

Chromic acid technique. In this case, chromic acid was used to fix the lipids to the tissues before paraffin-embedding. For that, the protocol described by Tracy and Walia (2002) was followed in detail[4]. As chromic acid fixes but does not stain lipids, lung sections were later stained with Oil O Red and counterstained with Mayer´s hematoxylin for fat demonstration.

For the Oil Red O staining technique, various organic solvents, as propylene glycol or isopropyl alcohol, have been proven to successfully dissolve Oil Red O to stain fats[44]. In the present study both solvents were tested, and finally, isopropyl alcohol was selected. This solvent allowed the proper staining of fat emboli since propylene glycol caused artifacts, i.e. Oil Red O clumps, hindering the proper evaluation of the tissue.

Frozen section technique. Formalin-fixed lung sections were rinsed in phosphate-buffered saline (PBS, pH = 7.4) for two days, and then cryoprotected in 30% sucrose solution in PBS (pH 7.4) at 4 °C to avoid freezing artifacts until lung samples sank. Then lung sections were cut with a cryostat and placed in Vectabond-treated glass slides, to ensure a proper adherence of the lung sections. To demonstrate the lipids, slides were stained with Oil Red O, dissolved in isopropyl alcohol, and counterstained with Mayer's hematoxylin.

Microscopic analysis. We evaluated the area occupied by fat emboli in lung sections treated for the different techniques using light microscopy (Olympus BX51).

For this purpose, each lung tissue section was divided in "N" number of 100× magnified microscopic fields (MF) (Ocular: 10× and Objective: 10×) (100 MF = 1,920,000 pixels). Then, a Bootstrap analysis was carried out to determine the number of 100 MFs ("n") that needed to be considered for each tissue section. Depending on the total lung tissue section area the Bootstrap analysis yielded results between 14 and 20 100 MFs, that were randomly selected and photographed[42]. As a result, for each animal we ended up having an "n" number of 100 MFs (photographs), each of them with an area, in pixels, occupied by fat emboli. Fat emboli were recognized as black/red droplets primarily in the lumen of capillaries and small- and medium-sized arteries. The area occupied by fat emboli in each of the 100 MFs' fields studied was determined manually in number of pixels using ImageJ software (1.48 v, Wayne Rasband, National Institute of Health, USA). Manual selection was carried out as not all the emboli were homogeneously stained and not all the black, in the osmium tetroxide technique; or red areas, in the frozen and chromic acid techniques, were fat emboli.

Statistical analysis. To compare the capacity of fat emboli detection of the three techniques, the proportion of fat emboli in the lung section studied was calculated for each techniquefollowing the equation:

$$FE\ proportion = a/(n \times A)$$

Where a was the total pixels occupied by fat emboli in the lung section studied, calculated as the sum of the pixels occupied by fat emboli in the 'n' 100 MFs´ fields studied, n was the number of 100MFs studied and A was the area occupied by each 100MFs (=1,920,000 pixels).

The Wilcoxon signed-rank test (a non-parametric test for two related samples) was used to assess if the mean rank between the OsO_4 and the chromic acid, on one hand; and the OsO_4 and the frozen section techniques, on the other hand, differ. Statistical significance was set at $p < 0.05$. Data were analyzed using the R package, version 3.3.1[45].

Evidence of ethical approval. 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.

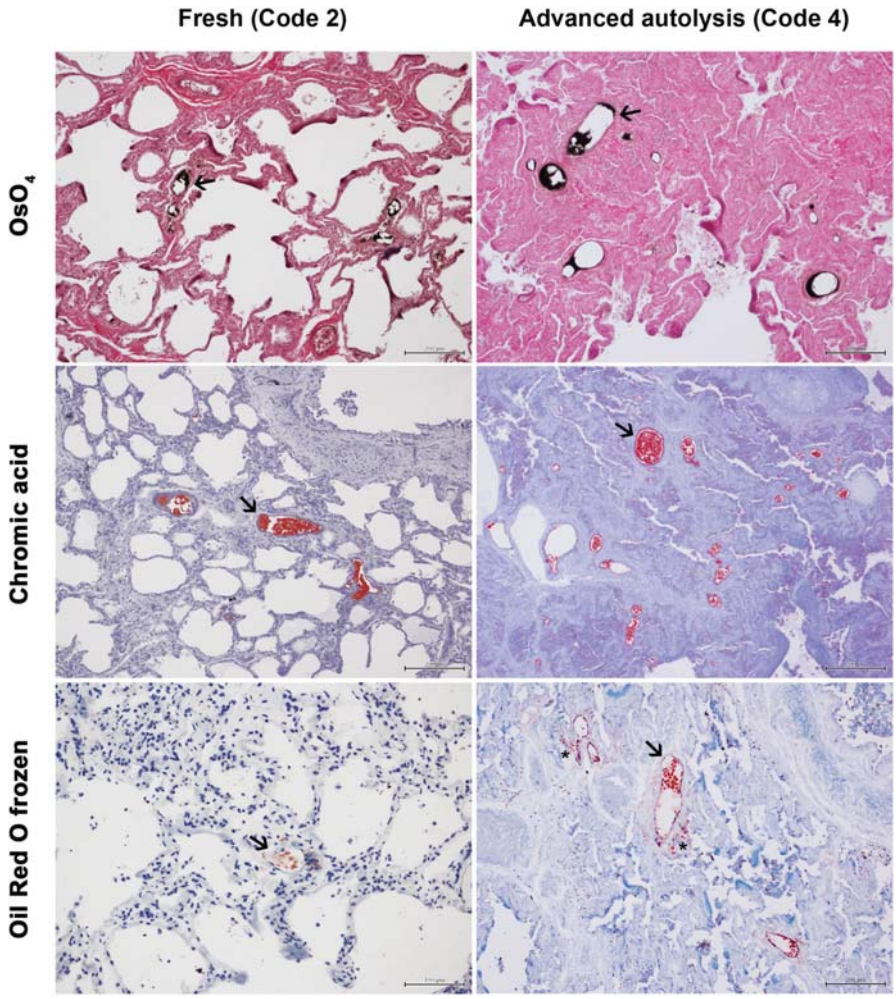

Figure 1. Comparison of the OsO_4, chromic acid and Oil Red O frozen-section techniques to detect fat emboli in the lung. Left images correspond to a fresh sperm whale and right images to an advance decomposed sperm whale. Examples of fat emboli are pointed out with arrows. Asterisks in the right-bottom image point to the surpassing vessel's limit. Bars = 200 µm, except in the left-bottom image where Bar = 100 µm.

Results

Among the eight animals studied, 75% presented an advanced (n = 5) or very advanced (n = 1) autolysis, and two cases were fresh or presented moderate autolysis. The three techniques were able to detect fat emboli in the lungs of the animals studied, even in carcasses presenting an advanced autolysis (Fig. 1). These emboli were mainly present in medium and small caliber intrapulmonary arteries and/or obliterating arterioles.

In the OsO_4 technique, the background presented a light to dark pink coloration, erythrocytes were very apparent in bright pink and cellular nuclei in purple, while fat emboli were stained in black. In the chromic acid and frozen section techniques, lipids appeared in red while the background (including erythrocytes and cellular nuclei) presented different shades from blue to purple, in the chromic acid technique; and from light blue (background) to dark blue (capillaries and nuclei) in the frozen section technique. The three techniques kept the tissue morphology although tissue components were more easily recognized at lower magnifications in the OsO_4 technique, as they stained in different colors, being the chromic acid, the technique showing the least differentiation (Fig. 1).

When referring to the fat emboli, in the OsO_4 and chromic acid techniques, although the center of the emboli was usually lost, the emboli borders appeared well fixed and stained. The center of the emboli was best preserved in the chromic acid technique. In the frozen section technique, the fat emboli detected were of lower quality, generally presenting diffuse staining and surpassing vessels' limits (Fig. 1).

No significant differences in the fat emboli proportion could be detected when comparing the OsO_4 technique to both: the chromic acid ($p = 0.4609$) and the frozen section ($p = 0.7998$) techniques.

Discussion

Fat embolism constitutes a pathological finding that has been extensively described in association with various processes. It is a very common finding, especially after traumatic injuries, and it is usually clinically asymptomatic[46] but can occasionally lead to a clinical disorder known as fat embolism syndrome (FES)[3,46].

In cetaceans, as well as in other species, including humans, the technique traditionally chosen to assess histologically fat embolism has been OsO_4. However, this technique has proven to be acutely toxic, requiring the implementation of extreme measures that should be strictly followed when working with it.

It is also important to highlight that, in the last years, health professionals (including veterinary pathologists and technicians) have been asked to follow new improved biosecurity measures in the field and laboratory diagnostic activities. Among these regulations, the implementation of histochemical techniques implying a low or no risk for the staff working in Veterinary Forensic Labs constitutes a requirement[47]. Considering this, a gradual transition to less toxic and more commonly used reagents, to minimize the risk of exposure, must be carried out.

With this objective in mind, chromic acid and Oil Red O frozen techniques were tested to assess fat embolism in ship-strike sperm whales´ lungs and compared to OsO_4, since they are less toxic and require reagents commonly used in different laboratory protocols. Results showed that both techniques preserved properly the lung tissue morphology and were able to histologically demonstrate fat emboli in the lungs of sperm whales presenting different decomposition codes that were kept in formaldehyde for years, as OsO_4 demonstrated in a recent study[42].

When assessing quantitatively the capacity of each technique for fat emboli detection, no significant differences were detected when comparing the area occupied by fat emboli in the OsO_4 to the other two techniques. However, the sample size was small and thus, the power to detect differences was low. Thus, future studies with a higher number of individuals should be carried out to enhance the statistical power. To our knowledge, this is the first study comparing quantitatively the detection capacity of the three techniques. There is just one previous study that attempted to compare OsO_4 and Oil Red O frozen section techniques for fat emboli detection[18]. Conclusions of this study showed that tissues treated with OsO_4 showed a higher degree of staining compared to the Oil Red O frozen section technique when small quantities of fat were present. When comparing these results to ours, it should be highlighted that: first, in the present study fat emboli were especially present in small size arteries and arterioles and not in capillaries, thus, the capacity of fat emboli detection when minute amounts of fat were present could not be compared among techniques; and secondly, while in the present study fat emboli area, followed by statistical analysis, is used for comparison between techniques, Abramowsky and colleagues followed the Mason's 1 to 4 scale, based on the number of emboli present in the tissue[16], which is a much less accurate methodology that scores fat emboli based on the observer personal criteria rather than in an objective quantitative grading system.

Apart from being necessary to make objective comparisons among techniques, the proper assessment of the amount/area of fat emboli present in a tissue section (fat embolism proportion) is essential as the implications of those emboli in the individual's death may vary significantly among grades. Considering this, the present study method has not only efficiently compared the techniques studied, but it constitutes an objective grading system that can be implemented in further studies aiming to properly assess fat embolism severity.

Among the three techniques, OsO_4 and chromic acid showed a superior fat emboli-staining quality compared to the frozen section technique, which is the least toxic. In the former ones, the intensity of the staining was superior and the emboli showed well-defined borders allocating them precisely within arteries and arterioles contrary to the third technique, in which lipids tended to move during the tissue processing as they were not previously immobilized (fixed). These differences were already noticed by Abramowsky and colleagues when comparing the OsO_4 and the Oil Red O frozen technique[18]. Better preservation of the emboli center in the chromic acid compared to the OsO_4 technique could be explained because the osmium only reacts with unsaturated fats, being unable to demonstrate saturated fats[48]. In the chromic acid technique, this limitation may be resolved by infiltrating the formalin-fixed tissue with an emulsion of a polyunsaturated fatty acids (linoleic acid) in ethylene glycol. These unsaturated lipids partition into the tissue lipids, enriching the number of unsaturations reacting with the chromic acid[4]. This advantage, together with its higher capacity of penetration, its more common use in laboratories, and its lower price, make chromic acid an interesting alternative to OsO_4 for histological fat emboli detection.

Further histochemical studies on fat embolism using other microscopy techniques, like confocal microscopy, will probably benefit from the features and possibilities of modern and more complete equipment. Other techniques, such as immunohistochemistry, have been recently described for fat embolism assessment. In these cases, although fat deposits are lost in the process of paraffin-embedding, the finding of fibrinogen and platelets around those empty spaces has been proposed as a proof of intravital fat embolism[49]. However, this should be taken carefully as in cases of decompression sickness, platelet[50–52], and less frequently fibrinogen[50], aggregation around the gas bubble surface has been demonstrated as well. In cetaceans, gas and fat embolism have been jointly described in beaked whales stranded in association with sonar activities[38,39], thus, techniques able to histologically demonstrate those fat emboli are essential. Considering this, immunohistochemistry analysis for fat embolism assessment should always be taken as a complementary diagnostic tool, especially useful when fresh frozen or fixed samples are not available, and not as a substitute of techniques able to visualize the fat emboli.

In conclusion, the three techniques were proven to be valuable for the diagnosis of fat embolism in cetacean tissues, even in decomposed tissues and after long periods in a fixative solution. Although quantitative differences could not be established, the chromic acid technique showed a higher quality in fat emboli staining than the Oil Red O frozen technique. The chromic acid technique also stands as a good alternative to OsO_4 due to its

equivalent capacity to detect fat emboli, or even superior when considering the preservation of the emboli center. It is also commonly used in other laboratory procedures and has minimal costs when compared with the expensive OsO_4. Although in the present study the different techniques were tested in sperm whales, these results are of application in human and animal forensic medicine.

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Author contributions

A.F. and Y.BdQ.: conceptualization. M.Arb., S.S., E.S., Y.BdQ., M.Arr and A.F.: sampling. E.S. and S.S.: protocol standardization. M.Arr, Y.P.-S.: laboratory analyses. A.S.: data analyses. A.F.: funding. MArr: writing. All authors: review and editing. A.F. and Y.BdQ.: supervision.

Competing interests

The authors declare no competing interests.

Additional information

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Lipids of lung and lung fat emboli of the toothed whales (Odontoceti)

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Lipids are biomolecules present in all living organisms that, apart from their physiological functions, can be involved in different pathologies. One of these pathologies is fat embolism, which has been described histologically in the lung of cetaceans in association with ship strikes and with gas and fat embolic syndrome. To assess pathological lung lipid composition, previous knowledge of healthy lung tissue lipid composition is essential; however, these studies are extremely scarce in cetaceans. In the present study we aimed first, to characterize the lipids ordinarily present in the lung tissue of seven cetacean species; and second, to better understand the etiopathogenesis of fat embolism by comparing the lipid composition of lungs positive for fat emboli, and those negative for emboli in *Physeter macrocephalus* and *Ziphius cavirostris* (two species in which fat emboli have been described). Results showed that lipid content and lipid classes did not differ among species or diving profiles. In contrast, fatty acid composition was significantly different between species, with C16:0 and C18:1ω9 explaining most of the differences. This baseline knowledge of healthy lung tissue lipid composition will be extremely useful in future studies assessing lung pathologies involving lipids. Concerning fat embolism, non-significant differences could be established between lipid content, lipid classes, and fatty acid composition. However, an unidentified peak was only found in the chromatogram for the two struck whales and merits further investigation.

Lipids are biomolecules present in all living organisms playing structural, metabolic, and endocrine roles. They are classified into two major groups: Polar (glycerophosphatides and sphingosylphosphatides), and nonpolar lipids (acylgycerols, waxes, sterols such as cholesterol, sterol esters, and free fatty acids)[1].

Common lipids are present across the animal kingdom; however, there is also high diversity present in certain lipid types, mainly to meet specialized functions[1]. As an example of this, toothed whales (odontocete cetaceans) are the only mammals that deposit waxes within adipose tissues[2–7]. Blubber is a specialized hypodermis that constitutes the primary storage for metabolic energy in cetaceans[8]. Although in most species it is composed of triacylglycerols, it is dominated by waxes in the deepest diving toothed whales (i.e., families *Ziphiidae*, *Kogiidae*, and *Physeteridae*)[7,9,10]. All toothed whales have another set of specialized adipose depots called acoustic fats (melon in the forehead region and mandibular fats in and around the lower jaws), which are cranial fat bodies that participate in the transmission and reception of the sound[11,12]. The acoustic fats are composed of endogenously synthesized waxes and triacylglycerols, usually containing branched fatty acids and fatty alcohols[3,4,7,9,13].

Apart from their physiological roles, lipids can also be involved in a great number of pathologies. One of them is fat embolism, which has been extensively described in human and animal science mainly associated with traumatic processes[14–17], and it is defined as the mechanical obstruction of the lumen of blood vessels, mainly in the lung microvasculature, by circulating fat particles[17].

As in other species, fat embolism in cetaceans is not a physiological or common finding in their lungs[18] but related to various pathological entities as ship strikes, intra/interspecific traumatic interactions, or decompression-like sickness[18–22]. In traumatic cases (e.g., ship strikes and intra/interspecific interactions), the origin of fat emboli is thought to be bone marrow from fractured bones or from damaged soft tissues entering the bloodstream through torn venules[17,23], as it has already been demonstrated in other species[24–28].

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Fat embolism has been described in combination with gas embolism (decompression-like sickness) in deep-diving species from the family *Ziphiidae* (beaked whales) that stranded in temporal and spatial association with naval exercises[21,29]. In this case, the origin of fat emboli remains unclear, although the most supported hypothesis postulates that gas bubble formation within the tissue may disrupt lipid-rich tissue, causing the entry of fat emboli in the bloodstream[21]. This hypothesis is supported by the fact that nitrogen is five times more soluble in lipids than in water[30,31] and that in vivo formation of gas bubbles has been reported in the abdominal adipose tissue of mice under hyperbaric treatments[32]. Therefore, lipid-rich tissues can act as a nitrogen sink. Moreover, nitrogen solubility has been shown to be higher in blubber containing wax esters (i.e., beaked whales), than in blubber without[33]. Also, larger concentrations of shorter, branched-chain fatty acids and fatty alcohols (found in the acoustic fat bodies), together with an increased WE content, appear to increase nitrogen solubility[34]. All that was mentioned allows hypothesizing that waxes may have a crucial role in the genesis of fat emboli in cetaceans suffering decompression-like sickness.

To assess a pathological lipid composition in cetacean lungs and be able to identify fat emboli lipid composition accurately, prior knowledge of the typical lipid composition of these species' lung tissue is essential. However, data on lung tissue lipid composition in cetaceans are very limited. To our knowledge, lung tissue lipid composition has only been determined in a few cetacean species: *Stenella coeruleoalba* (striped dolphin)[35–37] and *Phocoena phocoena* (harbour porpoises)[38]. In contrast, most studies have focused on the lipid composition of lung surfactant in cetaceans[39–42] due to its primary physiological function involving alveolar stability to prevent lung collapse in air-breathing vertebrates[43–45]. The study of lung tissue composition instead of surfactant lipid composition is required for fat emboli assessment, as surfactant studies are performed based on pulmonary lavage while fat emboli are located within lung tissue vessels. Moreover, it would be interesting to assess if lung tissue lipid composition varies among distinct diving profiles (deep vs. shallow divers), considering the differences in nitrogen gas exposure likely experienced by deep versus shallow divers.

To provide these much-needed baseline data, the objectives of the present study were (1) to determine regular lung tissue lipid composition (lipid content, lipid class, and fatty acid composition) of cetacean species with distinct diving profiles (i.e., deep vs. shallow); and (2) to better understand the pathogenesis of fat embolism by determining the lipid composition of lungs known to be positive for fat emboli, and comparing those with lungs negative for emboli. In addition to determining the major lipid classes in the lungs, we also examined the polar lipid components in more detail as they are the major lipids in the lungs.

Material and methods

Sample collection. Lung samples were obtained from freshly dead animals, encountered floating or stranded, in the Canary Islands and Andalusia (Spain) between the years 2006–2019 (Table 1). Required permission for the management of stranded cetaceans was issued by the environmental department of the Canary Islands' Government, the Regional Government of Andalusia, and the Spanish Ministry of Environment. Only fresh dead animals (no bloating nor changes in coloration, edible meat)[46] were considered for the study as fatty acids start to degrade immediately after death, resulting in the loss of polyunsaturated fatty acids (PUFAs)[47]. Lung samples were obtained during standard necropsies and stored frozen at − 20 °C until the analysis. All tissues were imported into the United States under valid CITES permits.

Species were segregated in deep and shallow divers, considering deep divers as those species known to forage at depths deeper than 500 m (*Physeter*, *Ziphius*, *Mesoplodon*, *Globicephala*, and *Grampus*); all others (*Stenella* spp.) were considered shallow divers[48]. To select the animals included in this study, the presence/absence of lung fat emboli was previously evaluated for animals whose pathological findings made them suspect of having lung fat emboli (i.e., those that have been struck by vessels or those presenting a gas embolic syndrome)[18,21]. Lung fat emboli were positive for three *Z. cavirostris* (Cuvier's beaked whales) presenting gas embolic syndrome, and two *P. macrocephalus* (sperm whales) involved in ship strikes. Control (negative to lung fat emboli) animals were also included for both species (two controls per species) (Table 1). Animals negative for fat embolism from other species were also included in the study to assess lung lipid composition across species and diving profiles: *Mesoplodon densirostris* (Blainville's beaked whale) (n = 3), *Globicephala macrorhynchus* (short-finned pilot whale) (n = 3), *Grampus griseus* (Risso´s dolphin) (n = 2), *Stenella coeruleoalba* (n = 4), and *Stenella frontalis* (Atlantic spotted dolphin) (n = 4) (Table 1).

Lipid extraction and analysis. Total lipids were extracted from the lung samples using a modified Folch procedure[49,50] and reported as percent wet weight (wt%). Three replicates were analysed for each individual.

TLC–FID analysis. The main lipid classes in each of the lungs were identified and quantified by thin-layer chromatography with a flame ionization detector (TLC–FID) (Iatroscan MK-6s; Iatron Laboratories, Inc.: Tokyo, Japan). Samples were spotted on chromarods and developed in 94/6/1 hexane/ethyl acetate/ formic acid. Lipid classes were identified through the use of lipid class standards (Nu Chek Prep, Elysian, MN, USA), and quantified as % of total lipid mass (wt%) using a computer software Peak Simple (Peaksimple 3.29, SRI Instruments, Torrance, CA, USA). This analysis provided data on basic lipid classes: triacylglycerols, free fatty acids, cholesterol, and phospholipids. With TLC–FID, wax esters and sterol esters co-elute and thus, cannot be distinguished from one another.

HPTLC polar lipids. The lungs of two representative animals of each species (negative to fat embolism) were studied using high-performance thin-layer chromatography (HPTLC).

Polar lipid classes were separated, identified, and quantified using a CAMAG high-performance thin-layer chromatography (HPTLC) system (CAMAG Scientific, Switzerland). Samples were spotted on glass silica gel 60

Case number	Species	Age	Body condition	Location	Diving profile	Lung fat emboli	Cause of death
1	*Z. cavirostris*	Subadult	Good	Fuerteventura	Deep	Negative	Others
2	*Z. cavirostris*	Adult	Good	Lanzarote	Deep	Negative	Others
3	*Z. cavirostris*	Adult	Good	Andalusia	Deep	Positive	Gas and fat embolic syndrome
4	*Z. cavirostris*	Adult	Good	Andalusia	Deep	Positive	Gas and fat embolic syndrome
5	*Z. cavirostris*	Subadult	Fair	Andalusia	Deep	Positive	Gas and fat embolic syndrome
6	*P. macrocephalus*	Neonate	–	La Gomera	Deep	Negative	Others
7	*P. macrocephalus*	Calf	Good	Tenerife	Deep	Negative	Others
8	*P. macrocephalus*	Calf	Good	Tenerife	Deep	Positive	Ship strike
9	*P. macrocephalus*	Juvenile	Good	Gran Canaria	Deep	Positive	Ship strike
10	*M. densirostris*	Adult	Very poor	Fuerteventura	Deep	Negative	Others
11	*M. densirostris*	Adult	Poor	Fuerteventura	Deep	Negative	Others
12	*M. densirostris*	Adult	Fair	Fuerteventura	Deep	Negative	Others
13	*G. griseus*	Adult	Good	Fuerteventura	Deep	Negative	Others
14	*G. griseus*	Adult	Fair	Tenerife	Deep	Negative	Others
15	*G. macrorhynchus*	Subadult	Fair	Lanzarote	Deep	Negative	Others
16	*G. macrorhynchus*	Juvenile	Poor	Gran Canaria	Deep	Negative	Others
17	*G. macrorhynchus*	Calf	Poor	Tenerife	Deep	Negative	Others
18	*S. coeruleoalba*	Subadult	Good	Lanzarote	Shallow	Negative	Others
19	*S. coeruleoalba*	Adult	Fair	Tenerife	Shallow	Negative	Others
20	*S. coeruleoalba*	Adult	Poor	Tenerife	Shallow	Negative	Others
21	*S. coeruleoalba*	Subadult	Poor	Tenerife	Shallow	Negative	Others
22	*S. frontalis*	Adult	Fair	Lanzarote	Shallow	Negative	Others
23	*S. frontalis*	Juvenile	Fair	Tenerife	Shallow	Negative	Others
24	*S. frontalis*	Adult	Fair	Tenerife	Shallow	Negative	Others
25	*S. frontalis*	Subadult	Fair	Tenerife	Shallow	Negative	Others

Table 1. Biological and stranding data of the animals included in the present study.

F_{254} HPTLC plates (Merck 105642, Merck KGaA, Darmstadt, Germany) using a CAMAG ATS 4 autosampler. Standards were obtained from Avanti Polar Lipids (Alabaster, Alabama, USA) and Nu Chek Prep (Elysian, Minnesota, USA). Approximately 10 mg of total lipids were spotted per application. A separation method modified from Vitiello and Zanetta[51] was used: HPTLC plates were pre-developed in a solvent system of methyl acetate: isopropyl alcohol: chloroform: methanol: 0.25% aqueous KCl (10:10:10:4:3.6 by volume) for 10 cm. Then, spotted plates were developed for 7 cm in the solvent system described above. Any nonpolar lipid classes present in the sample did not migrate in the solvent system used and were therefore not quantified using this technique.

Developed plates were dried in the fume hood for 5 min, dipped in a copper sulfate/sulfuric acid revelation solution (16 g H_3PO_4 + 6 g $CuSO_4$ + 200 mL distilled H_2O), and heated at 160 °C for 20 min. Developed plates were then scanned using a CAMAG TLC Scanner 4 (under Tungsten light at 371 nm wavelength) and imaged under RT white light using a CAMAG TLC Visualizer 2. Lipid classes were identified using standards developed on the same plate as the samples and quantified using linear regression of the peak areas of standards.

Gas chromatography (GC) analysis. For GC analysis, fatty acids from total lipid extracts were esterified (obtaining total esterified fatty acids from free and esterified fatty acids) and converted to butyl esters (FABEs), rather than methyl esters (FAMEs) to avoid the loss of short-chain components due to their high volatility[52].

Fatty acids were separated and analysed by GC using a Thermo Trace 1310 GC with a flame ionization detector (FID) in a fused silica column (30 × 0.25 mm internal diameter) (Zebron ZB-FFAP; Phenomenex, Torrance, CA). Helium was used as the carrier gas, and the gas line was equipped with an oxygen and water scrubber. The following temperature program was used to separate fatty acids by carbon chain length: 65 °C for 2 min, then hold at 165 °C for 0.40 min after ramping at 20 °C/min, hold at 215 °C for 6.6 min after ramping at 2 °C/min, and hold at 250 °C for 5 min after ramping at 5 °C/min. Fatty acid peaks were identified based on commercial fatty acids (Nu-Chek Prep, Inc., Elysian, MN) and known samples, in which fatty acids not present in the commercially available standards were identified based upon peak identification performed on a Thermo Trace Ultra GC/Polaris Q MS (Thermo Fisher Scientific, courtesy S. Budge, Dalhousie University) using a similar column. Peak identification was manually confirmed for each run. Peaks were then integrated using appropriate response

factors[53] with the Chromeleon GC software (ver. 7.2.7; Thermo Scientific, Waltham, MA, USA), providing quantification in weight percentage (wt%) for later transformation in mole% based on known molecular weights of all fatty acids present. Unknown fatty acids were assigned the molecular weight of the FABE next to them. Each fatty acid was described using the nomenclature A: BωX, where A is the number of carbon atoms, B is the number of double bonds, and X is the position of the double bond closest to the terminal methyl group.

Statistical analysis. The software PRIMER, vers. 7 (Plymouth Routines in Multivariate Ecological Research, Primer-E, Ltd., Ivybridge, U.K.), a non-parametric multivariate approach that allows the inclusion of percentage data[54], and SPSS, vers. 26.0 (SPSS, Inc., Chicago, IL) software were used for statistical analysis. In SPSS, non-parametric statistics were performed due to the small sample size[55]. Among them, the Mann–Whitney U or the Kruskal–Wallis tests for two or more than two independent samples were used. If Kruskal–Wallis test values were statistically significant, post hoc test adjusted to Bonferroni corrections were performed. Statistical significance was set at $p < 0.05$.

Due to the small sample size, the power to detect differences was lower than desired for all the comparison analyses performed in this study.

Lipid content. Lipid content data (wt%) was compared among species and between diving profiles in SPSS. Moreover, animals within the species *P. macrocephalus* and *Z. cavirostris* were tested for significant differences due to the presence of fat emboli.

Lipid classes and FA signatures. Lipid classes and all fatty acids were first compared between positive and negative animals to fat embolism within the *Z. cavirostris* and *P.macrocephalus* species using SPSS software. As no significant differences were found either for lipid class or fatty acid composition between positive and negative animals in both species, all animals of both species were considered in further statistical analysis.

For PRIMER analysis, only FAs at ≥ 1 mol% in at least one of the lungs analysed (n = 32) were considered for statistical analysis. When a particular fatty acid was not detected in a sample, its concentration was changed from zero to 0.005%. This value was below the minimum detectable level (0.01%), but it was not so small to result in extreme outliers[56]. Resemblance matrices on untransformed data were generated based on Bray–Curtis dissimilarity. Nonmetric multidimensional scaling (MDS, 25 re-starts, Kruskal scheme 1) plots were produced to compare overall lipid profiles of samples. MDS plots placed samples within a two-dimensional space based on the resemblance matrix. As a result, samples that appeared closer together in the two- dimensional space exhibited more similar lipid profiles. A 2D stress value, ranging from 0 to 1, is generated as an output. Low-stress values indicate high reliance on the model, and stress values lower than 0.2 were assumed to indicated confidence in the placement of samples relative to each other[57]. Cluster analysis was used to analyse similarity levels among species.

Analyses of similarities (ANOSIM, one-way, max. permutations = 999) were performed to determine the effect of species and diving profile on lipid class profiles and fatty acid signatures. The null hypothesis of this test is that no differences exist among the groups compared. The global R ranges from 0 to 1, with higher values indicating greater deviation from the null hypothesis. One-way similarity percentages analysis (SIMPER, one-way, based on Bray–Curtis similarity, cut-off percentage = 90) was conducted on all samples, if the analysis of similarities was significant, to determine the lipids classes/fatty acids that contributed the most to the differences observed between groups. Then, lipid classes/fatty acids explaining most of the differences were analysed for significant differences between/among groups in SPSS.

Different fatty acid ratios were calculated considering all fatty acids identified in the lung (n = 98). These were saturated (SFAs) to total unsaturated (MUFAs) (SFAs/(MUFAs + PUFAs)); monounsaturated (MUFAs) and polyunsaturated to total fatty acids (MUFAs/Total FAs and PUFAs/Total FAs); monounsaturated related to polyunsaturated (MUFAs/PUFAs); and the ratio between omega-six and omega-three polyunsaturated FAs (PUFA w6/w3). Ratios between animals positive and negative to fat embolism within the *Z. cavirostris* and *P. macrocephalus* species were compared statistically. As no significant differences were found, all animals of both species were considered in the statistical comparison of ratios among species.

Evidence of ethical approval. 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.

Results

Lipid content. The mean lipid content of the lung was between 0.91 and 3.43 wt% (Table S1). Although there was some variation, no significant differences in lung lipid content were detected among species ($p = 0.127$) or diving profiles ($p = 0.601$). Besides, no significant differences were detected when comparing positive and negative animals to fat embolism in *P. macrocephalus* ($p = 0.394$) and *Z. cavirostris* ($p = 0.689$).

Lipid class composition. In all lung samples, the lipid classes identified by TLC–FID were (from higher to lower concentrations): phospholipids (50.7–78.5 wt%), cholesterol (10.2–34 wt%), sterol/wax esters (0.8–22 wt%), free fatty acids (0.5–15.2 wt%), and triacylglycerides (0–5.7 wt%) (Table S1, Fig. 1). The wide percentage ranges indicate that there were inter-individual differences in lung lipid classes. In SPSS, non-significant differences in lipid classes were found between the absence and presence of fat emboli in both *Z. cavirostris* and *P. macrocephalus* (all p values > 0.05).

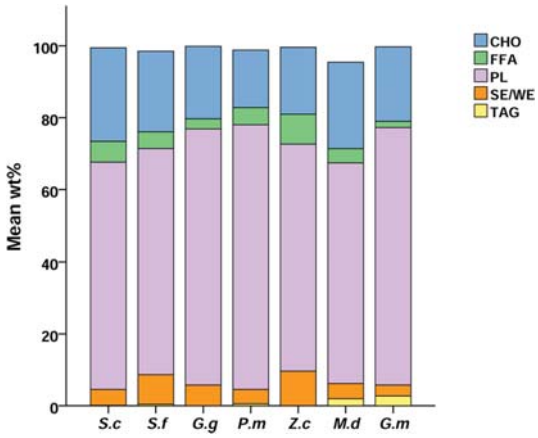

Figure 1. Mean wt% of the lipid classes present in the lung of the seven cetacean species studied. The lipid classes were: CHO = cholesterol, FFA = free fatty acids, PL = phospholipids, SE/WE = sterol esters and TAG = triacylglycerols. The species are: *S.c* = *S. coeruleoalba*, *S.f* = *S. frontalis*, *G.g* = *G. griseus*, *P.m* = *P. macrocephalus*, *Z.c* = *Z. cavirostris*, *M.d* = *M. densirostris*, and *G.m* = *G. macrorhynchus*.

Grouping by species, nonpolar lipid percentages among the different species ranged from 25.3 ± 5.2 wt% and 36.6 ± 10.1 wt%, and phospholipids from 61.2 ± 10.6 wt% to 73.5 ± 4.5 wt%. ANOSIM test determined that lipid classes presented non-significant differences among species ($p = 0.453$, global R = − 0.004), and between diving profiles ($p = 0.232$, global R = 0.06).

The most common phospholipids identified by HPTLC in the lung of the animals studied were glycerophosphatides (fatty acid linked to glycerol via an ester bond), and sphingosylphosphatides (fatty acid linked to sphingomyelin via an amide bond). In the first group, phosphatidylcholine (PC) was the most abundant, followed by phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) (Table S2). Within the second group, sphingomyelin was also present in high quantities (Table S2).

Fatty acid composition. Ninety-eight different FA were identified in the lungs, although only thirty-two were present at proportions ≥ 1 mole% in at least one of the lungs analysed. Samples were dominated by fatty acids of at least 16 carbons, with SFAs and MUFAs generally showing the highest percentages (Table 2). The most abundant fatty acids were the SFAs palmitic (C16:0) and stearic (C18:0) fatty acids; the MUFA oleic (C18:1n-9) fatty acid; and the PUFAs arachidonic (C20:4ω6), eicosapentaenoic (20:5ω3), and docosahexaenoic (C22:6ω3) fatty acids. Branched-chain components (< 5.5 mole%) were present in very low proportions. Of these, isolauric acid (*i*-C12:0) was present in the two species from the family *Ziphiidae* in particular (Table 2).

Differences in fatty acid composition between absence and presence of fat emboli in both *Z. cavirostris* and *P. macrocephalus* were not significant (all p values > 0.05).

The nMDS plot 2D stress value was 0.12 (< 0.2), indicating that the model was confident in the placement of the points (Fig. 2). The ANOSIM revealed non-significant differences in the fatty acid composition between diving profiles ($p = 0.08$; R = 0.149) but between species ($p = 0.001$, global R = 0.526).

The cluster analysis revealed that the lung fatty acid composition of the different species studied was over 65% similar. All the individuals from the family *Ziphiidae* were enclosed in a cluster revealing a fatty acid composition similarity of over 75% (Fig. 2A). The rest of the species were enclosed in another 75%-similarity cluster except for a *P. macrocephalus* neonate, found in a separate 75% similarity cluster (Fig. 2A).

SIMPER analyses revealed that the three fatty acids that contributed the most to the similarity within a single species were: C16:0 (17.1–39.1 mol%), C18:1ω9 (19.1–34.2 mol%), and C18:0 (12.5–18.3 mol%), being their cumulative effects between 58.4 and 74.8 mol%. At the same time, C16:0 and C18:1ω9, were the most influential in separating the fatty acid signatures among species. They were always among the top contributors to the dissimilarities between species (C16:0 (5.5–32.9%) and C18:1ω9 (4.5–26.2%)) with a cumulative difference effect between 16.6 and 50.0% (Fig. 2B). Significant differences were found among species ($p < 0.001$), as individuals from the Family *Ziphiidae* had higher levels of C18:1ω9 and lower values of C16:0 than the other groups (Fig. 2B).

The statistical comparison of ratios revealed non-significant differences between animals positive and negative to fat embolism for *Z. cavirostris* and *P. macrocephalus*, which was expected as non-significant differences were observed between fatty acids related to fat emboli. In the comparison among the seven species, significant differences were found between *S. frontalis* and *M. densirostris* in the MUFAs/Total FA ratio ($p = 0.039$); and between *P.macrocephalus* with both *Z. cavirostris* ($p = 0.036$) and M. densirostris ($p = 0.039$) for FAs/Total FAs ratios.

The chromatogram analysis revealed the presence of an unknown fatty acid (Unknown FA1) at 5.96 min in the lungs of the two *P. macrocephalus* positive to fat embolism, but it was not present in the individuals negative

Mean mole% ± S.D	Z. cavirostris (n = 5)	P. macrocephalus (n = 4)	M. densirostris (n = 3)	G. griseus (n = 2)	G. macrorhynchus (n = 3)	S. coeruleoalba (n = 4)	S. frontalis (n = 4)
C10:0	0.36 ± 0.38	0.02 ± 0.02	0.08 ± 0.10	0.01 ± 0.00	0.02 ± 0.00	0.05 ± 0.04	0.02 ± 0.01
i-C11:0	0.62 ± 0.59	nd	0.03 ± 0.04	nd	nd	0.01 ± 0.01	0.02 ± 0.01
i-C12:0	0.87 ± 0.83	0.00 ± 0.01	0.22 ± 0.28	0.01 ± 0.00	0.00 ± 0.01	0.01 ± 0.01	0.03 ± 0.03
C14:0	0.84 ± 0.18	4.27 ± 2.98	0.96 ± 0.23	1.60 ± 0.68	0.93 ± 0.19	1.26 ± 0.17	1.51 ± 0.26
i-C15:0	0.24 ± 0.18	0.07 ± 0.02	0.29 ± 0.21	1.75 ± 2.04	0.10 ± 0.07	0.62 ± 0.15	0.22 ± 0.14
C15:0	0.20 ± 0.02	0.63 ± 0.26	0.22 ± 0.02	0.86 ± 0.29	0.41 ± 0.02	0.73 ± 0.07	0.93 ± 0.14
i-C16:0	0.08 ± 0.04	0.07 ± 0.03	0.10 ± 0.08	0.62 ± 0.75	0.07 ± 0.04	0.44 ± 0.20	0.18 ± 0.07
C16:0	14.30 ± 2.06	33.91 ± 5.74	14.61 ± 1.17	29.81 ± 0.14	22.29 ± 2.09	23.79 ± 2.64	26.12 ± 4.73
C17:0	0.59 ± 0.13	0.66 ± 0.38	0.54 ± 0.06	0.48 ± 0.67	0.80 ± 0.15	0.67 ± 0.09	1.13 ± 0.19
C18:0	15.18 ± 2.16	10.89 ± 1.73	14.80 ± 1.63	12.11 ± 1.32	13.68 ± 1.34	11.51 ± 1.27	14.52 ± 2.26
TOTAL SFA	**35.59 ± 4.51**	**51.41 ± 5.39**	**33.87 ± 1.38**	**49.25 ± 2.79**	**39.79 ± 1.50**	**40.99 ± 3.48**	**46.14 ± 7.44**
C12:1d	0.00 ± 0.01	0.27 ± 0.54	0.11 ± 0.19	0.12 ± 0.16	nd	nd	nd
C16:1ω9	1.66 ± 0.63	1.79 ± 0.41	0.81 ± 0.16	1.12 ± 0.48	0.59 ± 0.33	1.08 ± 0.10	0.27 ± 0.19
C16:1ω7	2.78 ± 0.90	3.70 ± 3.89	2.87 ± 0.18	3.20 ± 2.93	2.70 ± 1.66	3.04 ± 0.84	2.10 ± 0.75
C18:1ω11	0.39 ± 0.23	0.35 ± 0.25	1.87 ± 0.70	nd	0.23 ± 0.38	0.34 ± 0.13	0.02 ± 0.04
C18:1ω9	25.72 ± 4.11	17.47 ± 2.81	29.20 ± 2.43	18.00 ± 2.57	23.09 ± 2.79	16.42 ± 0.76	15.74 ± 1.85
C18:1ω7	2.29 ± 1.79	1.40 ± 0.78	3.01 ± 0.23	2.49 ± 0.95	2.86 ± 0.32	2.11 ± 0.25	2.45 ± 0.67
C20:1ω11	0.73 ± 0.33	0.19 ± 0.16	1.30 ± 0.40	0.26 ± 0.05	0.56 ± 0.30	0.30 ± 0.09	0.07 ± 0.05
C20:1ω9	2.69 ± 0.88	1.16 ± 0.65	2.33 ± 0.21	1.55 ± 0.20	2.82 ± 0.44	2.72 ± 0.87	1.05 ± 0.49
C22:1ω11	0.66 ± 0.46	0.13 ± 0.09	0.82 ± 0.33	0.09 ± 0.00	0.31 ± 0.18	0.35 ± 0.13	0.06 ± 0.04
C24:1ω11	1.32 ± 0.78	0.74 ± 1.45	1.11 ± 0.79	0.76 ± 0.14	1.84 ± 0.24	2.87 ± 2.55	4.20 ± 4.27
C24:1ω9	0.87 ± 0.64	0.67 ± 0.09	1.32 ± 0.11	1.61 ± 0.69	1.27 ± 0.30	1.65 ± 0.10	1.11 ± 0.42
TOTAL MUFA	**41.19 ± 6.69**	**29.29 ± 5.02**	**46.21 ± 1.75**	**30.75 ± 8.36**	**37.78 ± 3.05**	**32.32 ± 3.36**	**28.34 ± 3.19**
C16:2ω4	1.11 ± 0.61	1.60 ± 0.69	1.36 ± 0.74	1.08 ± 0.15	0.55 ± 0.04	0.65 ± 0.41	0.89 ± 0.45
C16:4ω1	0.27 ± 0.60	nd	0.32 ± 0.55	nd	nd	0.02 ± 0.02	nd
C20:2ω6	0.22 ± 0.11	0.23 ± 0.19	0.15 ± 0.13	0.11 ± 0.01	0.35 ± 0.15	0.51 ± 0.27	0.80 ± 0.63
C20:4ω6	5.72 ± 3.34	5.82 ± 1.67	5.24 ± 4.63	4.17 ± 5.89	9.98 ± 1.18	8.17 ± 1.05	5.20 ± 3.98
C20:5ω3	5.26 ± 2.39	3.25 ± 1.46	2.54 ± 0.51	4.03 ± 2.73	2.98 ± 0.48	4.34 ± 0.69	2.68 ± 1.35
C22:4ω6	0.37 ± 0.21	0.66 ± 0.16	0.59 ± 0.10	1.42 ± 0.29	1.17 ± 0.30	1.03 ± 0.19	1.23 ± 0.50
C22:5ω3	1.55 ± 0.36	1.40 ± 0.36	1.14 ± 0.23	2.18 ± 0.67	1.73 ± 0.31	2.11 ± 0.36	1.89 ± 0.48
C22:6ω3	3.24 ± 1.91	3.18 ± 1.34	2.63 ± 0.46	4.18 ± 1.74	3.17 ± 0.19	3.64 ± 0.56	5.04 ± 1.96
TOTAL PUFA	**19.74 ± 4.58**	**17.91 ± 4.59**	**16.31 ± 5.21**	**18.57 ± 10.42**	**21.28 ± 2.39**	**22.27 ± 2.49**	**20.09 ± 6.73**
Unknown FA2	1.42 ± 2.25	0.03 ± 0.06	1.30 ± 2.24	nd	nd	1.00 ± 0.91	1.39 ± 1.63
Unknown FA3	0.43 ± 0.42	0.15 ± 0.11	0.77 ± 0.66	0.56 ± 0.26	0.00 ± 0.01	0.28 ± 0.29	0.24 ± 0.29
Unknown FA5	1.28 ± 0.76	0.60 ± 1.09	1.08 ± 0.92	0.63 ± 0.29	0.94 ± 0.15	2.64 ± 2.33	3.40 ± 3.38
TOTAL UNKNOWN FA	**3.48 ± 2.79**	**1.39 ± 1.27**	**3.03 ± 2.28**	**1.43 ± 0.73**	**1.16 ± 0.15**	**4.43 ± 2.70**	**5.44 ± 4.09**
SFA/ (MUFA + PUFA)	**0.59 ± 0.12**	**1.10 ± 0.20**	**0.54 ± 0.06**	**1.00 ± 0.10**	**0.75 ± 0.09**	**0.98 ± 0.28**	**0.68 ± 0.04**
MUFA/TOTAL FA	**0.41 ± 0.07**	**0.29 ± 0.05**	**0.46 ± 0.02**	**0.31 ± 0.08**	**0.32 ± 0.03**	**0.28 ± 0.03**	**0.38 ± 0.03**
PUFA/TOTAL FA	**0.20 ± 0.05**	**0.18 ± 0.05**	**0.16 ± 0.05**	**0.19 ± 0.10**	**0.22 ± 0.03**	**0.20 ± 0.07**	**0.21 ± 0.02**
MUFA/PUFA	**2.24 ± 0.86**	**1.75 ± 0.61**	**3.11 ± 1.31**	**2.12 ± 1.64**	**1.48 ± 0.30**	**1.56 ± 0.62**	**1.80 ± 0.32**
PUFA n-6/n-3	**0.78 ± 0.56**	**0.94 ± 0.17**	**1.04 ± 0.61**	**0.54 ± 0.25**	**1.01 ± 0.11**	**0.88 ± 0.24**	**1.49 ± 0.05**

Table 2. Fatty acid composition from total lipids (total esterified fatty acids) of the lungs of seven cetacean species. Fatty acid values are given in mean mole% ± S.D. Only fatty acids that showed a proportion of over 1 mole% in at least one of the animals were included in the table (n = 32). Values of total saturated (total SFA), monounsaturated (total MUFA), polyunsaturated (total PUFA), unknown (totally unknown) fatty acids, and ratios are shown as well (in bold) and were calculated from the total fatty acids (n = 98) identified in the lung. As there were no significant differences in the fatty acid composition of animals positive/negative to fat emboli, all animals from these two species were considered in the table (*Z. cavirostris* n = 5 and *P. macrocephalus* n = 4).

to fat embolism or in the other species analysed. The percentage concentrations of this fatty acid were 0.6 mol% (Case 8) and 0.8 mol% (Case 9) (Fig. S1). The structure of this fatty acid is currently unknown.

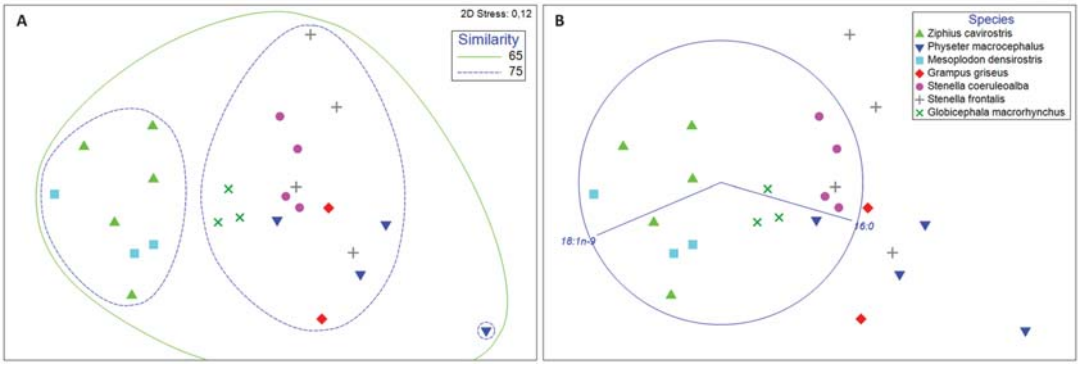

Figure 2. nMDS plot (Euclidean distance) of untransformed fatty acid proportions (n = 32) in the lungs of the animals studied. They are separated by species, with a different symbol and colour, and plotted individually based on the similarity of fatty acid signature. (**A**) Contours are a result of cluster analysis and represent 65% and 75% similarity. (**B**) Vectors indicate the top two FAs identified by SIMPER analyses that distinguish FA profiles among species.

Discussion

Lipid content. Lipid content showed intra-individual variability and was not associated with the diving profile or the presence of lung fat embolism. Lung lipid content ranged between 1 and 2% in all the species studied. These values agree with previous studies in striped dolphins[35,36,58], and other species, such as humans[59]. A study carried out by Storelli and colleagues in 1999, reported lower lipid percentages in the lung of a Cuvier´s beaked whale and a Risso's dolphin (0.4 and 0.66%, respectively). Nevertheless, it should be highlighted that these previous values are based on just one animal of each of these two species, and the condition of the carcasses in this study was not reported[58].

Lipid classes composition. Lipid classes in lung tissue did not differ between positive and negative for fat embolism, or among species. Lipid classes present in cetacean lungs were the same as those previously reported in the literature for various species, as in the study published by Clements about the lipid composition of 11 terrestrial and aquatic vertebrate species[60]. Moreover, amounts of nonpolar lipids (cholesterol, cholesterol ester, FFA, and TAG) and phospholipids in the lungs of the cetacean species studied here were very similar to those described in other species such as dogs[61], rats[62], and humans[63].

Among polar lipid classes, most studies have focused on lung surfactant composition, which is a material produced and secreted by the lung epithelium to assist in alveolar stability. Lung surfactant is composed of phospholipids (90%), specifically phosphatidylcholine, and proteins (10%)[45]. Phospholipids present in the surfactant are also present in the lung tissue, as part of lamellar bodies, although their proportions are different (in lavage fluid: 75–90% and in lamellar bodies: 60%)[43]. For example, phosphatidylcholine is the dominant phospholipid class in both surfactant and lung tissue, but its content is higher in the surfactant than in the lung tissue. As well, phosphatidylglycerol is the second most abundant phospholipid in the surfactant, but its contribution is much lower in the lung tissue. In contrast, phosphatidylethanolamine is abundant in the lung tissue but is present in low amounts in the surfactant[43].

Although we do not know how much of the polar lipids identified in the lungs of the animals in the current study were from surfactant or for the lung tissue itself, phosphatidylcholine and phosphatidylethanolamine constituted the major phospholipid classes identified, as it is the case for lung tissue. Other phospholipids also present in substantial amounts were sphingomyelin, phosphatidylserine, and finally, phosphatidylinositol, being this constancy kept among several species such as dogs, humans, chickens or bovines among others[64].

Fatty acid composition. The fatty acids identified in the lungs were generally similar among the different species studied, with only a few of them presenting significant quantitative differences. In all the species, except the two species from the family *Ziphiidae*, SFAs showed the highest percentage, followed by MUFAs and finally PUFAs, in agreement with previous studies in striped dolphins[36,37].

The most abundant fatty acids in the lung of all the animals studied were those commonly encountered in most animal tissues. These were the SFAs palmitic (C16:0) and stearic (C18:0) acids, the MUFA oleic acid (C18:1ω9), and the PUFAs arachidonic (C20:4ω6), eicosapentaenoic (C20:5ω3), and docosahexaenoic (C22:6ω3)[1,6,65]. Among the identified fatty acids, palmitic and oleic acids showed the highest proportion in all the lungs. The same results were observed in previous studies of the lipid composition of the lung in several vertebrate species, including sea lions, marine turtles, and humans[37,60].

Although the most prevalent fatty acids present in the lung seemed to be conserved among species, there were significant differences in the fatty acid profile among species. *M. densirostris* and *Z. cavirostris* (family *Ziphiidae*) showed a higher amount of the monoene oleic acid and a lower amount of the saturated palmitic acid compared

to the other species studied. These differences explain the grouping of these two species in a separate cluster from the other species, including some deep-diving species, and their differences in SFAs/Total FAs and MUFAs/Total FAs compared to the rest of species. For our purposes, with the species in this study, we cannot separate the effects of the diving regime from those of taxonomy; this question deserves further attention.

Other fatty acids present in the lungs of the various species described by Clements[60], such as myristic acid (C14:0) or the C16:1 group, were also present in our animals in similar amounts. The C18:2 group was present in the lung of most of the vertebrates studied by Clements, being the sea lion the species showing the lowest percent (2.5 molar percent)[60]. Similarly, in all the cetacean species included in this study, the percentages of this group were very low (0.35–0.86 mean mole%).

PUFAs constitute essential fatty acids in mammals, which means that they have to be obtained through the diet as mammals lack the needed desaturase enzymes to synthesize them[66]. Among them, arachidonic, eicosapentaenoic, and docosahexaenoic acids are present in higher amounts in tissues, as they constitute major components of membrane phospholipids throughout the animal kingdom. Arachidonic acid is the PUFA showing the highest content in the lungs of all the seven cetacean species included in the study. This finding agrees with previous studies in striped dolphins that concluded that this fatty acid was the most important not only in lungs but in all the other tissues studied, including melon, cerebrum, liver, or muscle[36,37,67]. The high ω6:ω3 ratio (≈1:1) in the lungs of the toothed whale species included in this study has also been described in different tissues of *S. coeruleoalba*, being more similar to land mammals than to other marine species, where the ratio is lower (as marine lipids are rich in ω3 fatty acids[1]). Williams and colleagues have suggested that this could indicate that marine mammals have maintained the requirements of the ω6-fatty acid from their terrestrial ancestors[67].

Other fatty acids present in the lungs of the various toothed-whale species studied were branched-chain fatty acids (BCFA). They are endogenous fatty acids, derived from the catabolism of branched-chain amino acids[68]. Among them, short branched-chain fatty acids appear in high concentrations in the acoustic fats and blubber of some species of toothed whales[3,6,9]. Some of the most important ones are isovaleric acid (*i*-5:0) in *Delphinidae*[2,3,6,9,13,34], *Phocoenidae*[2,3], and *Monodentidae*[69] families; and isolauric acid (*i*-C12:0) in the acoustic fats of the *Ziphiidae* family. In delphinids, *i*-C5:0 has been detected, but in much lower quantities, in other tissues, such as liver and muscle, indicating a certain degree of synthesis and deposition of this BCFA in those tissues[2]. Despite its presence in various cetacean tissues, *i*-C5:0 was not present in the lungs of the different delphinid species included in the present study, in agreement with previous research in an adult porpoise[38].

In ziphiids, *i*-C12:0 constitutes a major fatty acid in melon and mandibular fat tissues[3,4,7,13], and it was also present, although in much lower quantities, in their lung tissue. In those species in which *i*-C12:0 was not detected in acoustic tissues, such as *P. macrocephalus* and delphinid species[4], *i*-C12:0 was present in negligible quantities (close to 0) or not present at all in the lung.

Considering all that was mentioned previously, fatty acids present in the lungs seem to be phylogenetically conserved across toothed whale species, with only small differences in the presence (*i*-C12:0) and proportion of specific fatty acids (C16:0 and C18:1ω9). This fact differs from other toothed whale tissues, like acoustic fats, where a robust phylogenetic diversity in the fatty acids present has been described[4].

Concerning fat embolism, differences in fatty acid composition or the presence of fatty acids characteristic from other tissues in the lungs of *P. macrocephalus* and *Z. cavirostris* positive to fat embolism, could not be established in this study. The only evidence of a potential signal of fat embolism was the presence of an unknown short-chain fatty acid at the beginning of the chromatogram of the two ship-strike *P. macrocephalus* (Fig. S1). Further research should be carried out to identify this fatty acid and see if a relationship with the fatty acid composition of the marrow from the fractured bone or the injured soft tissue of the struck animals can be established.

We suggest that future research should be focused on the identification of fatty alcohols in the lungs of beaked whales presenting a gas embolic pathology. Since wax esters consist of a fatty alcohol esterified to a fatty acid, the presence of fatty alcohols indicates that component was sourced exclusively from a wax ester. Wax esters are the major components in both acoustic fats and the blubber of species from the family *Ziphiidae*[3,4], and nitrogen has proven to be highly soluble in them[33,34]. Thus, the presence and identification of fatty alcohols exclusively in the lungs positive to fat embolism would provide invaluable knowledge to the etiopathogenesis of fat embolism in these animals. As sperm whales´ blubber is mostly composed of waxes[10], and this tissue, together with muscles and bones, is one of the most affected when a ship-strike occurs[19,20], identification of fatty alcohols in the lungs of struck animals would be of great interest, as well.

To conclude, lipid content and lipid profiles (lipid classes and fatty acids identified) seem to be conserved among lung tissue in the animal kingdom, and variability in fatty acid amounts seem to be driving the differences among species in this tissue, e.g., oleic acid in beaked whales. The knowledge provided in this study on regular lung tissue lipid composition in cetaceans will be extremely useful in future studies aiming to assess lung pathologies involving lipids.

Data availability

All the samples reported in this work are 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

A.F. and Y.B.d.Q.: conceptualization. M.A., Y.B.d.Q., I.F., F.C., and M.J.C.: sampling. M.A. and H.L.G.: laboratory analyses. M.A. and H.L.G.: data analyses. A.F.: funding. M.A.: writing. All authors: review and editing. H.L.G., Y.B.d.Q., H.N.K., and A.F.: supervision.

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Competing interests

The authors declare no competing interests.

Additional information

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Lipids of lung and lung fat emboli of the toothed whales (Odontoceti)

Supplementary information

Table S1. Percentage of lipid content and lipid class composition (wt%) of the lung of the different species studied. Values given are the mean percent content of total lipid ± S.D. As there were no significant differences in the fatty acid composition of animals positive/negative to fat emboli, all animals from these two species were considered in the table (*Z. cavirostris* n=5 and *P. macrocephalus* n=4)

Species	Lipid content	TAG	FFA	Sterol Ester	Cholesterol	Phospholipids
	Mean wt% ± S.D.					
Z. cavirostris	2.33 ± 0.68	0.01 ± 0.03	8.32 ± 6.22	9.61 ± 7.45	18.61 ± 7.40	63.03 ± 10.45
P. macrocephalus	1.75 ± 0.20	0.57 ± 0.76	4.76 ± 2.34	3.97 ± 4.64	16.01 ± 1.02	73.50 ± 4.54
M. densirostris	1.55 ± 0.20	1.95 ± 1.18	3.99 ± 0.45	4.24 ± 3.83	24.07 ± 3.35	61.20 ± 10.60
G. griseus	1.43 ± 0.33	0.00 ± 0.00	2.81 ± 3.30	5.76 ± 1.42	20.18 ± 3.92	71.10 ± 8.70
G. macrorhynchus	1.42 ± 0.21	2.72 ± 2.62	1.75 ± 0.54	3.04 ± 0.72	20.71 ± 1.03	71.49 ± 3.92
S. coeruleoalba	1.66 ± 0.40	0.11 ± 0.15	5.79 ± 1.96	4.44 ± 1.43	26.05 ± 8.98	63.07 ± 8.46
S. frontalis	1.51 ± 0.54	0.45 ± 0.41	4.66 ± 4.45	8.21 ± 3.44	22.42 ± 8.08	62.74 ± 8.66

Table S2. Concentration (ug/mL) of the most important phospholipid classes identified in the lungs of the samples analysed. PC= phosphatidylcholine, PE= phosphatidylethanolamine, PI= phosphatidylinositol, PS= phosphatidylserine, Sphm= sphingomyelin.

Species	Case	PC	PE	PI	PS	Sphm
		Concentration (ug/mL)				
Z. cavirostris	1	738.5	520	346.2	537.2	483
	2	288.7	276.2	70.26	132.9	221.5
P. macrocephalus	6	681.3	458.9	435	499.8	313.7
	7	699.1	465.7	445	349.2	268.9
M. densirostris	11	695.2	501	160.4	335	502.4
	12	596.9	466.2	106.6	252.2	444.6
G. griseus	13	869.5	552.3	388.7	570.7	482.9
	14	468.4	299.4	100.1	283.7	415.9
G. macrorhynchus	15	759.2	514.9	257.4	380.5	525.4
	17	895	502.9	346.7	417.1	515.5
S. coeruleoalba	18	399.4	307.5	116.9	176.5	310.4
	21	531.6	350.8	182.6	237.6	290.7
S. frontalis	22	869.6	471.2	407.4	289.2	497.9
	25	807.6	416.3	307.8	260.6	487.5

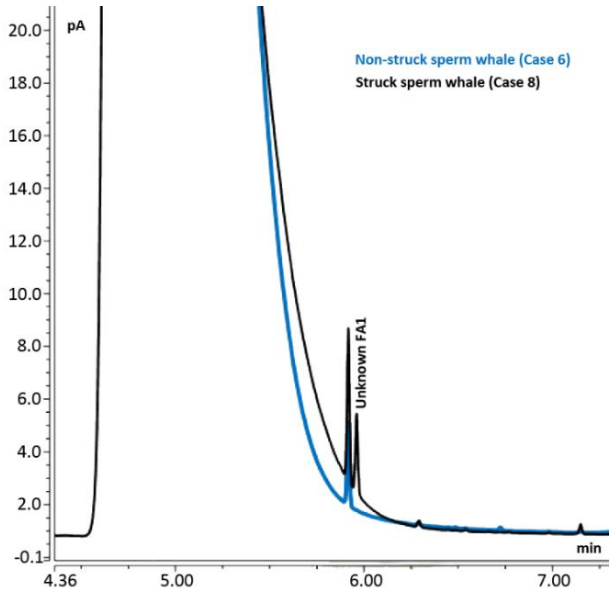

Fig. S1. Chromatogram inset of one struck sperm whale (case 8) and one non-struck sperm whale (case 6). At 5.96 min, presence of an unknown fatty acid (Labelled as "Unknown FA1") in the chromatogram of the struck sperm whale but not in the non-struck sperm whale.

4. CONCLUSIONS

1) The presence of fat emboli within lung vessels in histochemical studies constitutes reliable diagnostic evidence of antemortem ship-strikes events. Its assessment has proven to be especially important when other gross and microscopic trauma-related findings are not evident in the anatomopathological study, as it may happen when working with carcasses presenting an advanced decomposition status.

2) Most of the sperm whales (83%) stranded in the Canary Islands with evidence of ship strike were alive at the moment of the strike. Among them, calves were significantly more involved in ship strikes than other age categories, and the presence of fractures was significantly associated with these traumatic events. The data obtained in this study stress the importance of implementing proper mitigation measures to guarantee the survival of this species´ population in Canarian waters.

3) The histochemical techniques for lipid demonstration: OsO_4, chromic acid, and Oil red O frozen section, can accurately demonstrate histologically fat emboli in lungs presenting different decomposition codes kept in formaldehyde for long periods.

4) The chromic acid technique, tested for the first time in cetaceans´ lungs, stands as an excellent alternative to the commonly used but highly toxic OsO_4 technique for fat emboli detection. It shows an equivalent detection capacity to OsO_4, with even higher quality staining the emboli's entire center. Additionally, it has a lower cost, and it is regularly used in laboratory procedures.

5) Lung lipid content, lipid classes, and FAs seem to be phylogenetically conserved across toothed whale species, with only slight differences in the presence and the proportion of specific FAs.

6) The lung lipid composition (content, lipid classes, and FAs) of animals positive and negative to lung fat embolism was very similar. However, an unknown peak was found in the ship-strike sperm whales' chromatogram that merits further investigation.

5. RESUMEN EXTENDIDO

5.1. INTRODUCCIÓN

En las últimas décadas, se ha producido una creciente preocupación científica y social sobre el impacto que las actividades humanas están teniendo en el medio marino, poniendo de manifiesto la necesidad de implementar políticas proambientales.

Dentro de los organismos marinos, los cetáceos constituyen excelentes bioindicadores de la salud de los océanos por varias razones: 1) son especies paraguas, es decir, al ocupar extensos hábitats, su protección permite a su vez la protección de especies marinas que ocupan el mismo entorno, 2) tienen una larga esperanza de vida y muchas de sus especies se encuentran en la cúspide de la cadena trófica, reflejando lo que ocurre en niveles inferiores de la misma, y 3) son especies bandera, es decir, especies carismáticas capaces de captar la atención del público y de las autoridades gubernamentales para la efectiva protección de la naturaleza (Hoyt, 2011; Prideaux, 2003).

Entre las principales amenazas de carácter antropogénico para las poblaciones de cetáceos a nivel mundial, destacan el sonido (p. ej.: el uso de sónares activos), las colisiones con embarcaciones, la ingestión de plástico y la captura accidental en redes de pesca, entre otros (de Vere et al. 2018). En este trabajo de investigación nos centraremos en las dos primeras.

El uso de sónares antisubmarinos durante maniobras navales militares, ha sido asociado a varamientos masivos atípicos. Estos varamientos implican a más de dos animales, excluyendo madre y cría, y en ocasiones a varias especies, que llegan a una misma costa en un período corto de tiempo (Geraci and Lounsbury, 2005), siendo los zifios (Familia Ziphiidae) las especies de cetáceos más afectadas (revisado en Bernaldo de Quirós et al., 2019).

El estudio *postmortem* de estos animales, reveló la presencia de lesiones compatibles con burbujas de gas tanto en el parénquima como en los vasos sanguíneos de varios órganos vitales (Fernández et al., 2005; Jepson et al., 2003). Debido a las similitudes de esta nueva entidad patológica con el

síndrome de enfermedad descompresiva, descrito en buceadores humanos cuando la presión de los gases disueltos en el organismo, especialmente el nitrógeno (N_2), excede la presión absoluta externa, se le denominó "Patología embólica grasa-gaseosa" o "Decompression-like sickness" (enfermedad similar a la descompresiva) (Vann et al., 2011). Posteriores investigaciones, revelaron que las burbujas de gas presentes en estos animales estaban compuestas mayoritariamente por N_2, principal gas causante de la enfermedad descompresiva (Bernaldo de Quirós et al., 2011).

Estos animales presentaban un embolismo graso, además del embolismo gaseoso, en varios órganos vitales (Fernández et al., 2005; Jepson et al., 2003). El embolismo graso se define como presencia de material lipídico en el interior de un vaso sanguíneo, pudiendo llegar a obstruir el flujo del mismo. Los émbolos de grasa pueden ser visualizados bajo el microscopio óptico mediante el uso de técnicas de fijación y tinción adecuadas. El órgano diana para su estudio es el pulmón, ya que su microvasculatura de pequeño diámetro, retiene la mayoría de los émbolos y actúa como un primer filtro para proteger la circulación sistémica de la llegada de los mismos (Watson, 1970).

El embolismo graso es un hallazgo patológico que ha sido ampliamente descrito en humanos y otros animales, especialmente relacionado con causas traumáticas, como la fractura de huesos largos (p. ej. Gupta and Reilly, 2007; Serota, 1984). En una minoría de casos, puede derivar en un cuadro clínico conocido como síndrome del embolismo graso, que incluye principalmente manifestaciones neurológicas, insuficiencia respiratoria y petequias, además de otros signos y alteraciones analíticas (ten Duis, 1997).

El embolismo graso en cetáceos, también ha sido descrito en ausencia del embolismo gaseoso, por lesiones traumáticas como las colisiones con embarcaciones o las interacciones intra e interespecíficas (Díaz-Delgado et al., 2018).

Por otro lado, las colisiones con embarcaciones constituyen un impacto antropogénico en las poblaciones de cetáceos, que se ha visto agravado en las últimas décadas por el aumento en el número y la velocidad de los barcos (Laist et al., 2001). Estas colisiones constituyen un peligro especialmente importante para poblaciones de cetáceos que se encuentran altamente amenazadas, como p. ej. la ballena franca del Atlántico Norte en la costa este del Océano Atlántico (Sharp et al., 2019). Para minimizar la amenaza que las colisiones suponen para estas poblaciones, y garantizar su preservación, es necesario determinar tanto el estado de conservación de las distintas poblaciones afectadas, como las áreas de mayor solapamiento entre rutas marítimas y diversidad de cetáceos, con el fin de establecer medidas de mitigación efectivas como pueden ser restricciones de velocidad o el cese del tráfico marítimo en áreas determinadas.

El número de estudios enfocados en el embolismo graso en cetáceos es muy limitado, en comparación con el embolismo gaseoso, habiéndose apenas avanzado en su diagnóstico, su significación forense y origen.

En esta tesis doctoral se abordarán los siguientes objetivos con el fin de proporcionar un mayor conocimiento sobre el embolismo graso, tanto aislado como asociado a embolismo gaseoso, en cetáceos víctimas de colisiones y enfermedad de tipo descompresivo respectivamente:

1) Determinar si la presencia de embolismo graso en los pulmones de cachalotes, varados en aguas canarias con evidencia de colisión, constituye una evidencia diagnóstica efectiva de trauma *antemortem*.
2) Desarrollar un método histológico cuantitativo para determinar de manera objetiva la abundancia de émbolos de grasa en el pulmón.
3) Testar y comparar la eficiencia de diferentes técnicas histoquímicas en el diagnóstico del embolismo graso.
4) Analizar la composición lipídica del pulmón de varias especies de cetáceos con dos subobjetivos: 1) determinar la composición

lipídica habitual del pulmón de cetáceos y 2) determinar la composición lipídica de los émbolos grasos, comparando la composición lipídica de pulmones positivos y negativos a embolismo graso.

5.2. PUBLICACIONES

5.2.1. EMBOLISMO GRASO EN CACHALOTES COLISIONADOS CON EMBARCACIONES

Las Islas Canarias son un archipiélago español caracterizado por una gran diversidad de cetáceos, con 30 especies identificadas en sus aguas (Tejedor and Martín, 2013). El cachalote, una de estas especies, está presente durante todo el año en aguas canarias, en mayor número durante la estación migratoria. Está catalogado como vulnerable por la Unión Internacional para la Conservación de la Naturaleza (Taylor et al., 2019), y es la especie más afectada por colisiones con embarcaciones en aguas canarias (Arbelo et al., 2013; Díaz-Delgado et al., 2018), debido principalmente al aumento del tráfico marítimo entre islas y a la incorporación de ferries de alta velocidad (≥ 30 nudos) (de Stephanis and Urquiola, 2006; Ritter, 2010). Este impacto se ve agravado por la filopatría femenina en estas islas, ya que la población de cachalotes en las Islas Canarias, se encuentra aislada genéticamente de las demás poblaciones de la misma especie del Atlántico Norte (Alexander et al., 2016).

Las principales lesiones macroscópicas observadas en estos animales, resultantes de la colisión con una embarcación, son de tipo inciso y/o contuso. Las lesiones de tipo inciso, causadas por partes cortantes del barco (p. ej. la quilla o la hélice), se caracterizan por la presencia de lesiones de carácter lineal, que frecuentemente afectan a planos inferiores (p. ej. musculatura esquelética) y que en los casos más extremos pueden llevar a la amputación de parte del cuerpo (Fig. 5.1). Cuando el impacto se produce con una parte no cortante del barco (p. ej. el casco del barco), el trauma es de tipo contuso. En este tipo de trauma es habitual la presencia de edema y hemorragias en la grasa hipodérmica o blubber, tejido subcutáneo, musculatura esquelética e incluso afección de órganos internos en los casos de mayor severidad (Campbell-Malone et al., 2008; Moore et al., 2013).

A nivel microscópico, las principales lesiones incluyen hemorragia, edema, reacción inflamatoria (Arbelo et al., 2013; Campbell-Malone et al., 2008; Díaz-Delgado et al., 2018; Moore et al., 2013), así como fragmentación, degeneración y/o necrosis de las fibras musculares (Sierra et al., 2014). Sin embargo, la diferenciación entre lesiones *antemortem* y cambios *postmortem*, puede constituir un verdadero reto cuando se trabaja con animales en un avanzado estado de descomposición (Campbell-Malone et al., 2008; Moore et al., 2013), como es el caso de más del 70% de los cachalotes varados en las costas canarias en los últimos 20 años (Arbelo et al., 2013; Díaz-Delgado et al., 2018). Es en estos casos, donde el embolismo graso puede constituir un hallazgo patológico de gran importancia. La razón de ello es que, en los estudios forenses, la presencia de émbolos de grasa en la microvasculatura pulmonar constituye una evidencia fehaciente de que la lesión se produjo antes de la muerte. Esto se debe a que cuando se produce un trauma, los adipocitos (células adiposas) de la médula ósea del hueso fracturado y/o del tejido adiposo circundante, entran al torrente sanguíneo a través de las vénulas desgarradas en el lugar de la lesión, llegando mediante el retorno venoso a la circulación pulmonar y quedando mayoritariamente atrapadas en su microvasculatura (Levy, 1990; Watson, 1970). Para que esas partículas de grasa lleguen al pulmón, es necesario el latido del corazón bombeando la sangre a los distintos órganos, al menos durante un período breve de tiempo (Armstrong et al., 1955; Mason, 1968; Saukko and Knight, 2004).

Fig. 5.1. Lesiones por colisión con embarcación en cachalotes (*Physeter macrocephalus*) varados en las Islas Canarias: A) Presencia de una incisión profunda, inmediatamente caudal a la cabeza, con fractura craneal asociada; B) Presencia de cortes lineales seriados, probablemente causados por la hélice de un barco, afectando a la piel y a los tejidos subcutáneo y muscular; C) Presencia de una incisión profunda en el flanco derecho del animal, caudal a la aleta pectoral, con exposición del tejido subyacente, perforación abdominal y evisceración, así como fracturas costales; D) Amputación completa de la columna vertebral a nivel de la última vértebra torácica.

Considerando todo lo mencionado anteriormente, en este estudio nos propusimos analizar los pulmones de cachalotes varados, con evidencias de colisión, en costas canarias entre los años 2000 y 2017, la mayoría de los cuales presentaba un avanzado estado de descomposición. Los objetivos fueron los siguientes: 1) determinar si la presencia de embolismo graso es un hallazgo habitual (fisiológico) en el pulmón de cachalotes o está relacionado con eventos traumáticos, 2) determinar si la presencia de embolismo graso en vasos pulmonares, es una evidencia diagnóstica útil para determinar si una

colisión fue *antemortem*; 3) si se puede relacionar la densidad del embolismo graso pulmonar con la severidad y la localización de las lesiones.

Para la realización de este estudio, se seleccionaron cachalotes que presentaban hallazgos patológicos compatibles con colisión con embarcación (n=16); y cachalotes "controles", es decir, sin evidencia de colisión (n=8). El tetraóxido de osmio (OsO_4) fue el reactivo empleado para demostrar histológicamente, en color negro, los émbolos de grasa en muestras de pulmones previamente fijadas en formol. A continuación, mediante microscopía óptica, se evaluó la presencia de émbolos de grasa en vasos pulmonares. Para ello se diseñó un sistema de cuantificación objetivo, en el cual se determinó y estudió para cada animal, un número "n" de campos microscópicos con un aumento 100X (Ocular 10X y Objetivo 10X) representativos del número total ("N") de campos. En cada uno de esos "n" campos seleccionados, se determinó el área en píxeles ocupado por los émbolos de grasa, mediante el uso de un software de conteo especializado.

A su vez, distintas variables como la edad, condición corporal, presencia/ausencia de fracturas y localización del trauma, entre otras, fueron estudiadas para determinar su asociación con la severidad del embolismo graso, basándonos en que, a mayor área ocupada por émbolos grasos, mayor es la severidad del embolismo graso en el pulmón.

En los pulmones de dos de los ocho cachalotes "controles", se hallaron émbolos grasos de manera esporádica. El informe anatomopatológico de ambos animales, reveló la presencia de traumas contusos de naturaleza desconocida, como pueden ser interacciones traumáticas entre animales de la misma o diferente especie (Díaz-Delgado et al., 2018), o un potencial varamiento activo (Bernaldo de Quirós et al., 2019), con las consecuencias anatómicas y fisiológicas que esto puede tener para un animal acostumbrado a flotar. Dado que el embolismo graso se halló también presente en animales control, se realizó un análisis de clasificación y regresión (CART) con el fin

de establecer un valor de corte, por encima del cual, la probabilidad de que el animal haya sido víctima de una colisión sea alta.

Los resultados de este estudio evidenciaron, en primer lugar, que el embolismo graso no es un hallazgo fisiológico o habitual en los pulmones de cachalotes, ya que no se detectaron en la mayoría de los animales "control". En segundo lugar, un 83% (13/16) de los cachalotes estudiados con evidencia de colisión con embarcación, tanto frescos como en avanzada descomposición, presentaron émbolos de grasa en su vasculatura pulmonar, principalmente en el lumen de arterias, arteriolas y capilares (Fig. 5.2). La presencia de embolismo graso pulmonar, indica que estos animales estaban vivos en el momento en que se produjo la colisión, y que el colapso cardiovascular no se produjo de forma inmediata, permitiendo la irrigación pulmonar durante un período de tiempo desconocido. Este porcentaje puede ser una subestimación del porcentaje real, ya que, la metodología del embolismo graso presenta algunas limitaciones como es el muestreo arbitrario de algunas porciones pulmonares, que deben ser interpretados con cautela, ya que pueden no ser representativos de todo el tejido pulmonar.

El análisis CART concluyó que si el valor del área de embolismo graso del percentil 75 del animal estudiado es mayor de 140 píxeles (valor de corte), la probabilidad de que el animal haya sufrido una colisión es alta. Si el valor del área ocupado por embolismo graso del percentil 75 es menor de 140 píxeles, pero el animal es una cría, también es asignado al grupo de colisión. Finalmente, si no se cumplen ninguna de las dos condiciones previas, el animal es asignado al grupo de no colisión. Así, el presente estudio propone que el área ocupada por embolismo graso, es un mejor estimador de severidad que la tradicional escala basada en el número de émbolos, ya que un mismo número de émbolos en dos secciones pulmonares pueden ocupar áreas diferentes, siendo el que presente una mayor área ocupada el de mayor severidad.

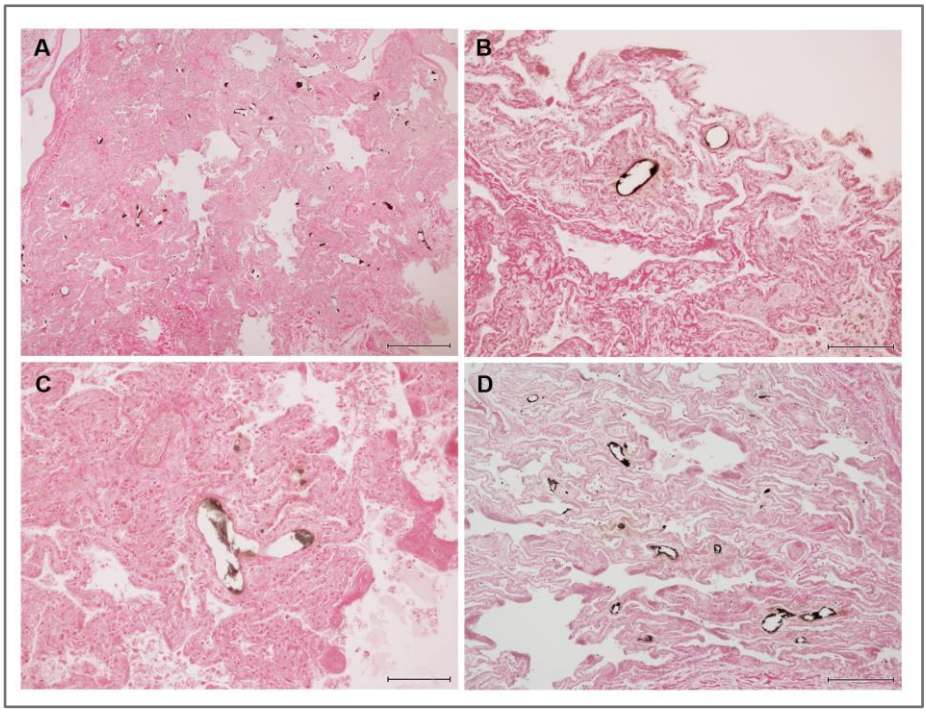

Fig. 5.2. Émbolos grasos teñidos de negro (OsO_4-positivos) en la microvasculatura pulmonar, principalmente en arteriolas, de cachalotes varados en costas canarias, con evidencia de haber sufrido una colisión con embarcación (A-D). Barra: A= 500 µm; B= 100 µm; C= 100 µm y F= 200 µm.

En relación a las distintas variables estudiadas, los resultados indicaron que las crías son significativamente más propensas que las otras categorías de edad a estar involucradas en colisiones, lo que puede deberse a su lenta natación o a su mayor permanencia en la superficie al no estar aun totalmente adaptadas al buceo (Laist et al., 2001; Papastavrou et al., 1989; Whitehead, 2009). A su vez, la presencia de fracturas estuvo asociada de manera significativa a las colisiones. Por último, no se pudo establecer una asociación entre variables relacionadas con trauma (p. ej. localización del trauma o número de fracturas) y la severidad del embolismo graso. Esto se pudo deber, entre otras causas, al bajo número muestral para un análisis estadístico adecuado, o bien a un colapso cardiovascular inmediato impidiendo la llegada de los émbolos al pulmón, explicando por qué en individuos con lesiones

traumáticas de mayor importancia, hay una menor severidad o ausencia total de embolismo graso pulmonar, como ya describieron en humanos Kinra y Kudesia (2004).

Para concluir, la presencia de émbolos grasos en el tejido pulmonar constituye un hallazgo diagnóstico fidedigno de colisión *antemortem*, especialmente importante cuando otras evidencias patológicas, tanto macro como microscópicas, no son concluyentes. Finalmente, el alto porcentaje de cachalotes víctimas de colisiones en aguas canarias, urge a la implantación de medidas de mitigación adecuadas para reducir las colisiones y garantizar la supervivencia de la población de cachalotes en estas aguas.

5.2.2. COMPARATIVA DE TRES TÉCNICAS HISTOLÓGICAS PARA LA DETECCIÓN DEL EMBOLISMO GRASO EN TEJIDO PULMONAR DE CETÁCEOS

Durante décadas se han utilizado diferentes técnicas para la demostración histológica del embolismo graso en microscopía óptica, electrónica y más recientemente, en microscopía confocal (p. ej. Fukui et al., 2006; Tracy and Walia, 2002; Turillazzi et al., 2008). Dado que la microscopía electrónica y confocal no son accesibles para muchos laboratorios diagnósticos ya que requieren de un equipamiento costoso y de personal muy bien entrenado para su manipulación, en este estudio nos hemos centrado en técnicas de microscopía óptica.

Las técnicas para la demostración histológica de lípidos en microscopía óptica, pueden basarse 1) en el corte en congelación de material fresco o previamente fijado en formol, o 2) en el trabajo con tejido incluido en parafina. En el caso de incluir el tejido en parafina, es necesario fijar los lípidos al tejido antes de su procesamiento ordinario, ya que los lípidos son solubles en los solventes empleados para embeber los tejidos en parafina. Cuando la técnica de tinción empleada parte de tejido cortado en congelación, no es necesario llevar a cabo esta fijación previa al no emplear solventes orgánicos en el procesado.

Entre las técnicas para la demostración del embolismo graso a partir de tejidos cortados en congelación, tanto de material fresco como formolado, destacan el Sudan Black B y el Oil Red O (Bancroft, 1975). Sin embargo, estudios previos sugieren que las técnicas en congelación, a pesar de suponer un menor riesgo para la salud, proporcionan una menor calidad morfológica y presentan una menor capacidad para detectar pequeñas cantidades de lípidos que las técnicas de demostración en parafina (Abramowsky et al., 1981; Carriel et al., 2017).

Entre las técnicas empleadas para la demostración lipídica en tejidos embebidos en parafina, destaca el OsO_4, empleado durante décadas debido a su gran calidad para demostrar histológicamente el embolismo graso (p. ej. Adams et al., 1967; Mudd et al., 2000) y que actúa al mismo tiempo como fijador y como teñidor, mostrando los lípidos en negro. Sin embargo, el OsO_4 es un reactivo extremadamente tóxico (United Nations, 2017), especialmente sus vapores, siendo necesario el seguimiento de estrictas normas de seguridad durante su manipulación. Otra técnica, descrita más recientemente para la demostración del embolismo graso en tejidos en parafina, es la técnica del ácido crómico (Tracy and Walia 2002). En ella, el ácido crómico se emplea para la fijación de los lípidos al tejido, y posteriormente se emplea el Oil Red O para teñir los lípidos de rojo. Esta técnica plantea algunas ventajas respecto al OsO_4 como son: su menor precio, su mayor capacidad de penetración en el tejido y una toxicidad ligeramente inferior. No obstante, el ácido crómico, al igual que el OsO_4, es un reactivo muy tóxico, además de cancerígeno, y por lo tanto debe ser manejado de manera segura (National Center for Biotechnology Information, 2005).

En la demostración histológica del embolismo graso, no sólo es importante la detección de los émbolos grasos, sino también su gradación objetiva con el fin de determinar la severidad del mismo. Esto es necesario, porque a mayor severidad, mayor es la probabilidad de que el embolismo graso esté involucrado en la muerte del paciente. Sin embargo, actualmente no existe una escala aceptada universalmente para la gradación del embolismo graso, sino diferentes escalas semicuantitativas basadas en el número de émbolos presentes en el tejido de estudio, como son las escalas de Falzi, Sevitt o Mason (Falzi et al., 1964; Mason, 1962; Sevitt, 1962). Entre las limitaciones de estos índices semicuantitativos, están que se asientan en el criterio personal del observador, que no tienen en cuenta el tamaño de los émbolos, y finalmente, que no consideran el área total del tejido estudiado, sugerido por algunos autores como necesario para una determinación adecuada del nivel de embolización (Busuttil and Hanley, 1994; Turillazzi et al., 2008). Por ello es necesario desarrollar técnicas de gradación cuantitativas y objetivas para la

adecuada determinación de la severidad del embolismo graso desde un punto de vista morfológico.

Finalmente, es necesario establecer si las diferentes técnicas tienen una capacidad similar de detección y cuantificación de los émbolos, con el fin de poder seleccionar la técnica más eficiente y de menor toxicidad. Sin embargo, en nuestro conocimiento, solo hay un estudio previo comparando la capacidad de detección del OsO_4 y la técnica de Oil Red O en congelación, basándose en el criterio personal del observador en lugar de una comparación cuantitativa apropiada (Abramowsky et al., 1981).

Considerando todo esto, los objetivos de este estudio fueron: 1) determinar la capacidad del ácido crómico para la demostración del embolismo graso en cetáceos, ya que a diferencia del OsO_4 y del Oil Red O en congelación, esta técnica no ha sido previamente empleada en cetáceos para este diagnóstico y, 2) comparar cualitativa (p. ej. intensidad de la tinción o localización precisa del émbolo en el vaso) y cuantitativamente (similar detección de émbolos de grasa) la capacidad para detectar émbolos de grasa del OsO_4, una de las técnicas más usadas, con respecto a las técnicas del Oil Red O en congelación y del ácido crómico.

Para ello, los pulmones de ocho cachalotes varados con signos de colisión y positivos a embolismo graso pulmonar (resultados del apartado 5.2.1 de esta tesis), fueron analizados en este estudio. Las muestras de pulmón formoladas de cada uno de los ocho animales, fueron tratadas para la demostración de émbolos de grasa mediante tres técnicas: OsO_4, ácido crómico y Oil Red O para corte en congelación. Con el fin de comparar la técnica del OsO_4, que es la más empleada, con las otras dos técnicas de la forma más precisa posible, las muestras de pulmón formoladas fueron cortadas a la mitad, siendo una de las caras espejo tratada con OsO_4 y la otra con ácido crómico. A continuación, la misma o diferente región pulmonar fue cortada a la mitad y una de ellas tratada con OsO_4 y la otra mediante corte en congelación con Oil Red O.

El área ocupada por émbolos de grasa en las secciones pulmonares estudiadas con cada una de las técnicas se evalúo, para cada animal, siguiendo la metodología desarrollada en el apartado 5.2.1 de esta tesis doctoral. En resumen, se determinó el área ocupada, en píxeles, por émbolos de grasa, en un número "n" de campos microscópicos, representativos del área tisular total para cada uno de los pulmones analizados.

La comparación entre las tres técnicas en relación a su capacidad de detección del embolismo graso, se calculó determinando para cada técnica la proporción de tejido ocupada por émbolos de grasa, respecto al área pulmonar estudiada mediante la siguiente ecuación:

$$Proporción\ de\ EG = {}^{a}/_{(n \cdot A)}$$

Donde "a" es el número de píxeles totales ocupados por émbolos de grasa en la sección pulmonar estudiada (calculado como el sumatorio de píxeles ocupados por émbolos grasos en los "n" campos microscópicos estudiados), "n" es el número de campos microscópicos estudiados y "A" es el área de cada uno de los campos microscópicos (100 MF = 1.920.000 píxeles). A continuación, se determinó si existían diferencias entre el rango medio del OsO_4 y del ácido crómico, por un lado, y del OsO_4 y la técnica de corte en congelación por el otro, mediante la prueba de rangos con signo de Wilcoxon (test no paramétrico para muestras relacionadas). La significación estadística se estableció en $p < 0,05$.

Los resultados indicaron que las tres técnicas son válidas para la detección histológica del embolismo graso, incluso en los casos en los que los animales presentaban un avanzado estado de descomposición (cinco de los ocho casos estudiados). Los émbolos grasos estaban presentes, fundamentalmente, en arterias de mediano y pequeño tamaño y en arteriolas (Fig. 5.3). A su vez, las tres técnicas preservaron de manera adecuada la morfología del tejido, siendo la técnica de OsO_4 la que permitía un mejor reconocimiento de las estructuras tisulares a pocos aumentos.

En las técnicas de OsO_4 y del ácido crómico, los émbolos presentaban bordes bien definidos siguiendo en muchos casos la luz vascular. La técnica del ácido crómico fue la que mostró una mayor preservación del centro del émbolo. En la técnica de corte en congelación, al no haber sometido a una fijación previa a los lípidos del tejido, los émbolos grasos presentaban menor calidad, mostrando una tinción difusa que sobrepasaba en muchas ocasiones los límites de los vasos (Fig. 5.3).

Estadísticamente no se detectaron diferencias significativas en la proporción de embolismo graso en el tejido estudiado entre las técnicas. No obstante, el poder estadístico de este estudio fue menor del deseado, debido al pequeño tamaño muestral.

Hasta donde conocen los autores, este es el primer estudio comparando cualitativa y cuantitativamente la capacidad de detección de embolismo graso de las tres técnicas descritas. Entre las principales conclusiones obtenidas, destaca que las tres técnicas son válidas para un adecuado diagnóstico del embolismo graso, incluso en tejidos que presentan un avanzado estado de descomposición y/o almacenados en solución formólica durante largos períodos de tiempo. Además, las tres técnicas mostraron una capacidad de detección del embolismo graso similar. Sin embargo, fueron el OsO_4 y el ácido crómico, las que mostraron una mayor calidad de tinción del embolismo graso, siendo el ácido crómico la técnica que permitió una mayor preservación del centro del émbolo. Considerando todo esto, concluimos que la técnica del ácido crómico constituye una buena alternativa al OsO_4 para la detección del embolismo graso, además de ser ligeramente menos tóxica, de menor coste y de uso habitual en muchos procedimientos de laboratorio.

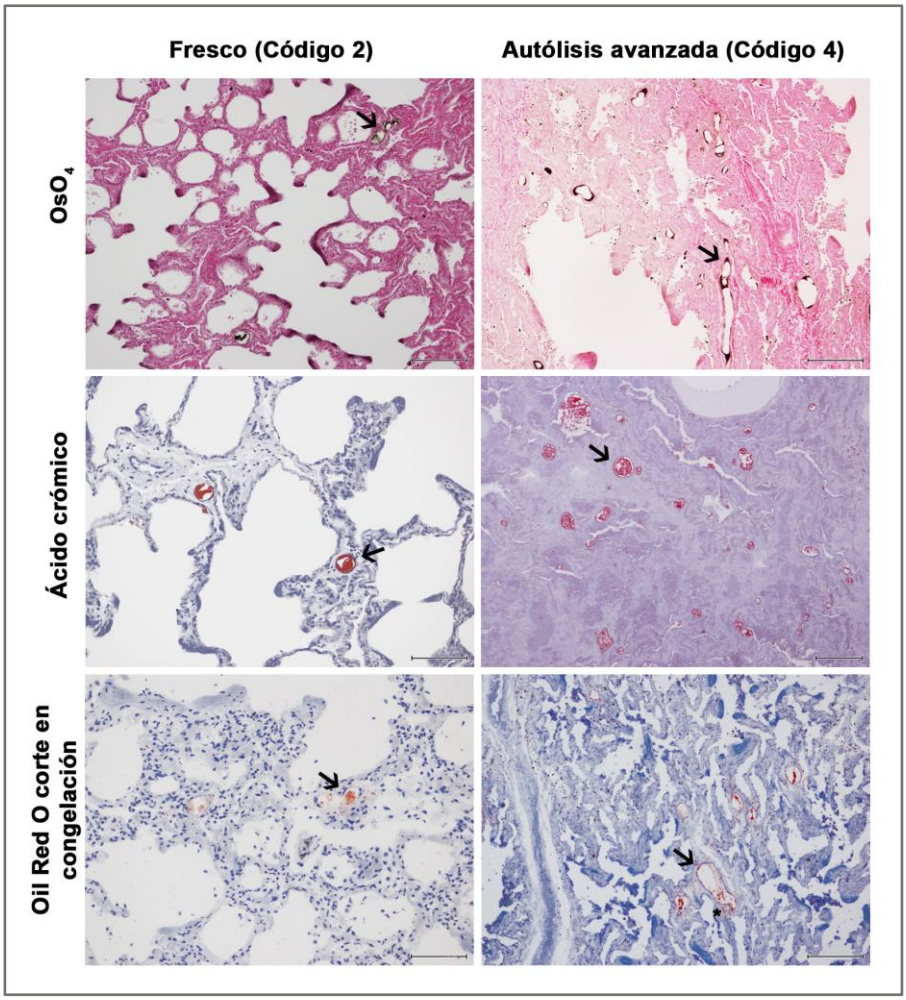

Fig. 5.3. Comparación entre las técnicas de OsO_4, ácido crómico y Oil Red O en congelación, para la demostración de émbolos de grasa en el tejido pulmonar. Las imágenes de la izquierda, corresponden a un cachalote fresco (código 2 en una escala sobre 5), mientras que las imágenes de la derecha, corresponden a un cachalote en avanzado estado de descomposición (código 4 sobre 5). Las flechas señalan algunos ejemplos de émbolos grasos. El asterisco en la imagen inferior derecha, indica un área en el que la tinción sobrepasa los límites del vaso sanguíneo. Barras= 200 µm, excepto las imágenes superior e inferior izquierda donde la barra= 100 µm.

5.2.3. LÍPIDOS PULMONARES Y EMBOLISMO GRASO PULMONAR EN CETÁCEOS ODONTOCETOS

Los estudios sobre la composición lipídica pulmonar en cetáceos son muy escasos, habiéndose centrado la mayoría de ellos en la composición del surfactante pulmonar (por ej. Miller et al., 2006; Spragg et al., 2004), encargado de la estabilidad alveolar mediante la reducción de la tensión superficial en la interfase respiratoria aire-líquido, evitando así el colapso pulmonar (Fisher, 2015).

Para poder ahondar en la composición lipídica en pulmones con embolismo graso, así como profundizar en el origen de esos émbolos de grasa, es necesario conocer previamente la composición lipídica estándar del tejido pulmonar en estos animales. Los estudios de la composición lipídica del surfactante pulmonar, basados en lavados bronquiales y bronquiolares, no son los adecuados para un posterior estudio del embolismo graso, ya que la presencia de lípidos en los mismos no se da únicamente en el embolismo graso (Vedrinne et al., 1992).

En cetáceos con patología embólica grasa-gaseosa (enfermedad similar a la descompresiva), la hipótesis más barajada para explicar el origen del embolismo graso es que las burbujas de N_2 se forman en tejidos ricos en lípidos, ocasionando la disrupción de estos tejidos y provocando la entrada de émbolos de grasa en el sistema circulatorio. Esta hipótesis se apoya en el hecho de que el N_2 es cinco veces más soluble en los lípidos que en el agua (Vernon, 1907; Weathersby and Homer, 1980), y en que se ha demostrado la formación de burbujas *in vivo* en tejido adiposo de ratones sometidos a tratamientos hiperbáricos (Lever et al., 1966). Siguiendo esta hipótesis, la composición lipídica del émbolo se asemejará a la del órgano dónde se ha originado. En el caso descrito en el apartado 5.2.1. de embolismo graso por colisión con barcos, la composición lipídica de los émbolos grasos deberá asemejarse a la del tejido adiposo traumatizado.

Los principales tejidos adiposos en cetáceos odontocetos son el blubber, el melón y la grasa mandibular. El blubber o grasa hipodérmica, constituye el principal reservorio energético en cetáceos (Iverson, 2009) y está compuesto por triglicéridos en la mayoría de las especies de cetáceos, salvo en los de buceo profundo (Familias Ziphiidae, Kogiidae y Physeteridae), en los cuáles está dominado por ceras (Koopman, 2007).

El melón y la grasa mandibular están implicados en la transmisión y recepción del sonido, formados principalmente por triglicéridos (tres ácidos grasos esterificados a un glicerol) y ceras (un ácido graso esterificado a un alcohol graso), ambos compuestos en su mayoría por ácidos y/o alcoholes grasos de cadena ramificada (revisado en Koopman, 2018).

Estudios previos, han demostrado que la solubilidad del N_2 es mayor en el blubber formado por ceras que por triglicéridos (Koopman and Westgate, 2012), y que la presencia de ácidos grasos y alcoholes grasos de cadena ramificada (en melón y grasa mandibular) junto a un mayor contenido en ceras, aumentan la solubilidad del N_2 (Lonati et al., 2015). Considerando todo esto, resulta lógico hipotetizar que las ceras puedan tener un papel clave en el origen del embolismo graso, tanto en cachalotes colisionados como en zifios con patología embólica grasa-gaseosa.

En este estudio se ha pretendido en primer lugar, ampliar el conocimiento previo de la composición lipídica pulmonar fisiológica (incluyendo contenido lipídico, clases lipídicas y ácidos grasos) de varias especies de cetáceos, cuyas circunstancias de varamiento no les hacía sospechosos de embolismo graso, y que incluían buceadores superficiales y profundos (inferior vs. superior a 500 m de profundidad) (Bernaldo de Quirós et al., 2012), debido a la diferente exposición al N_2 de ambos grupos. Las especies elegidas fueron los delfines listados y moteados, como representativos de buceo superficial, y los calderones tropicales, calderones grises, cachalotes y zifios de Cuvier y de Blainville, dentro del grupo de buceo profundo.

En este estudio se trató además de determinar la composición lipídica del embolismo graso. Para ello, se analizó y comparó la composición lipídica de tres zifios de Cuvier y dos cachalotes, positivos a embolismo graso (patología embólica grasa-gaseosa y colisión con embarcación, respectivamente), con la composición lipídica de pulmones negativos a embolismo graso de las mismas especies.

Los resultados indicaron que el contenido lipídico (1-3%), las clases lipídicas descritas (fosfolípidos, esteres de esteroles, ácidos grasos libres y triglicéridos), y los ácidos grasos más abundantes, son similares entre las distintas especies de cetáceos odontocetos, y a su vez, a los ya descritos en otros vertebrados (p. ej. Clements, 1971; Guitart et al., 1999; Toshima and Akino, 1972) indicando una conservación filogenética de la composición lipídica pulmonar. En referencia a los ácidos grasos estudiados, las dos especies de la Familia Ziphiidae (los zifios de Cuvier y de Blainville), presentaron una concentración significativamente mayor del ácido oleico (C18:1ω9) y menor del ácido palmítico (C16:0), en comparación al resto de las especies. Este hallazgo merece ser investigado en futuros estudios, con el fin de determinar si esta diferencia está relacionada con el hábito de buceo o con la taxonomía de las distintas especies.

Los ácidos grasos de cadena ramificada, frecuentes en melón y grasa mandibular, se encontraban presentes también en el pulmón en condiciones fisiológicas en proporciones bajas (< 6 mol%). Además, la ratio, próxima a uno, de ácidos grasos omega-6 / omega-3 en estos animales, es más similar a mamíferos terrestres que a las demás especies marinas, ricas en omega-3, sugiriendo que los mamíferos marinos han mantenido los requerimientos de omega-6 de sus antepasados terrestres (Williams et al., 1987).

En relación a la composición lipídica de los émbolos de grasa, no se detectaron diferencias estadísticamente significativas ni para contenido lipídico pulmonar, ni clases lipídicas, ni para composición de ácidos grasos entre los cachalotes y zifios positivos en comparación con los negativos a

embolismo graso. El único hallazgo relevante en este aspecto, fue la presencia de un ácido graso, que aún no ha podido ser identificado, al inicio del cromatograma de los dos cachalotes de colisión, y ausente en los cachalotes control y demás especies analizadas (Fig. 5.4). Es necesario continuar investigando con el fin de identificar este ácido graso, y tratar de relacionarlo con la composición lipídica de la médula ósea del hueso fracturado o tejido graso traumatizado.

Para seguir profundizando en la etiopatogénesis del embolismo graso en estos animales, consideramos que sería interesante que estudios futuros se centraran en la identificación de alcoholes grasos. Las evidencias para creer esto son que: 1) los alcoholes grasos se encuentran de manera exclusiva en las ceras, 2) el N_2 es altamente soluble en ceras, 3) los zifios con patología embólica grasa-gaseosa presentaban hemorragias en el melón y la grasa mandibular, tejidos compuesto en gran parte por ceras (Fernández et al., 2005) y 4) los cachalotes presentan un blubber dominado por ceras, siendo este tejido uno de los principales afectados tras una colisión con embarcación. No obstante, el escaso conocimiento que se tiene actualmente sobre el posible origen del embolismo graso en cetáceos, hace necesario considerar distintas líneas de investigación futuras

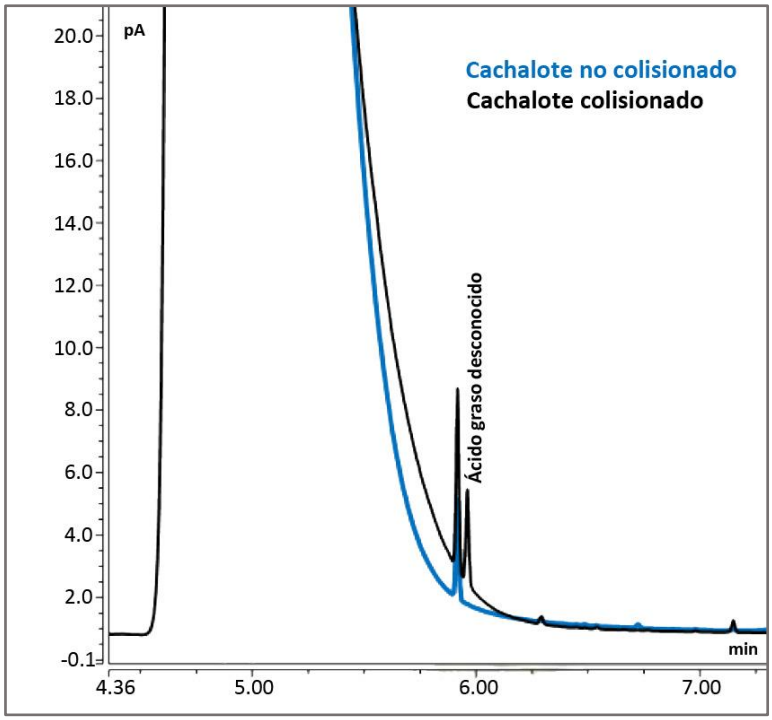

Fig. 5.4. Detalle del cromatograma de dos cachalotes, uno de ellos víctima de una colisión con una embarcación. En el minuto 5.96, se observó la presencia de un ácido graso desconocido, identificado como "ácido graso desconocido", en el cromatograma del animal colisionado (negro), pero ausente en el cromatograma del animal no colisionado (azul).

5.2.4. CONCLUSIONES

1) La presencia de émbolos grasos en el interior de los vasos pulmonares de cetáceos, ha demostrado ser una evidencia diagnóstica de gran utilidad, para determinar si una colisión fue *antemortem*. Su demostración histológica es determinante en el estudio anatomopatológico cuando otros hallazgos macroscópicos y microscópicos característicos de eventos traumáticos en vida no son evidentes, como ocurre en cuerpos que presentan un avanzado estado de descomposición.

2) Al menos, un 83% de los cachalotes varados en las Islas Canarias con evidencias de colisión con embarcación, estaban vivos en el momento en que se produjo el impacto. Las crías constituyeron la clase de edad significativamente más afectada. La presencia de fracturas en estos animales también estuvo relacionada significativamente con este tipo de evento traumático. Los resultados obtenidos en este estudio, reiteran la importancia de implementar medidas de mitigación adecuadas, para garantizar la supervivencia de la población de cachalotes en aguas canarias.

3) Las técnicas histoquímicas para la demostración de lípidos estudiadas: OsO_4, ácido crómico y el Oil Red O en congelación, han demostrado ser de gran utilidad en la manifestación histológica del embolismo graso en pulmones que presentan distintos grados de descomposición y que, además, han sido mantenidos en formol durante largos períodos de tiempo.

4) La técnica del ácido crómico, testada por primera vez en pulmones de cetáceos, constituye una alternativa adecuada al OsO_4, comúnmente utilizado, pero extremadamente tóxico, para la evidenciación histológica del embolismo graso. Su capacidad de detección es similar al OsO_4, incluso ligeramente superior en relación a la conservación del centro del émbolo, además de tener un coste inferior y ser de uso común en laboratorios.

5) En los pulmones de cetáceos odontocetos, el contenido lipídico, las clases lipídicas y las clases de ácidos grasos se han conservado

filogenéticamente, habiendo únicamente pequeñas diferencias en la presencia y proporción de un pequeño número de ácidos grasos.

6) La composición lipídica pulmonar (contenido, clases de lípidos y ácidos grasos) fue similar tanto en animales positivos como negativos al embolismo graso pulmonar. Sin embargo, se detectó un pico desconocido de manera exclusiva en los cromatogramas de los dos cachalotes víctimas de colisión con una embarcación, por lo que sería interesante realizar futuras investigaciones para la identificación de este ácido graso.

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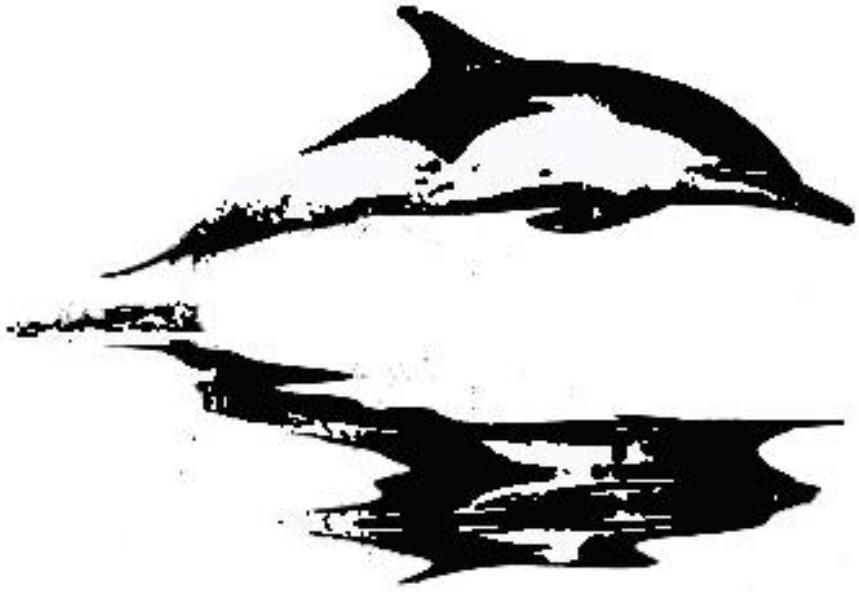
6. ABBREVIATIONS

ANOSIM	Analysis of Similarities
BCFA	Branched-Chain Fatty Acid
bpm	beats per minute
CART	Classification and Regression Tree / Análisis de Clasificación y Regresión
CHO	Cholesterol
CI	Confidence Interval
CO_2	Carbon dioxide
e.g.	for example
FA	Fatty Acid
FABE	Fatty Acid Butyl Ester
FAlc	Fatty Alcohol
FAME	Fatty Acid Methyl Ester
FE	Fat Embolism
FES	Fat Embolism Syndrome
FFA	Free Fatty Acid
Fig.	Figure / Figura
GC	Gas Chromatography

G. griseus; *G.g.*	*Grampus griseus*
G. macrorhynchus; *G.m.*	*Globicephala macrorhynchus*
HE	Hematoxylin & Eosin stain
HPTLC	High-Performance Thin-Layer Chromatography
i.e.	that is
M. densirostris; *M.d.*	*Mesoplodon densirostris*
MF	Microscopic Field / Campo microscópico
min	minute
mm	millimeter
MUFA	Monounsaturated Fatty Acid
N_2	Nitrogen / Nitrógeno
n-MDS	non-Metric Multidimensional Scaling
O_2	Oxygen
OsO_4	Osmium Tetroxide / Tetraóxido de osmio
PBS	Phosphate-Buffered Saline
p. ej.	por ejemplo
PL	Phospholipid

P. macrocephalus; *P.m.*	*Physeter macrocephalus*
PUFA	Polyunsaturated Fatty Acid
S. coeruleoalba; *S.c.*	*Stenella coeruleoalba*
S.D.	Standard Deviation
SE	Sterol Ester
SFA	Saturated Fatty Acid
S. frontalis; *S.f.*	*Stenella frontalis*
SIMPER	Similarity Percentage Analysis
TAG	Triacylglycerols
TLC-FID	Thin-Layer Chromatography with a Flame Ionization Detector
WE	Wax Ester
x	magnification
Z. cavirostris; *Z.c.*	*Ziphius cavirostris*
µm	micrometer
°C	Celsius degrees

7. APPENDIX



7.1. OIL RED O FOR FAT EMBOLISM DEMONSTRATION IN FROZEN OR PARAFFIN-EMBEDDED TISSUES

Pretreatment:

For frozen sections:

- Rinse formalin-fixed tissue sections in PBS (pH = 7.4) for two days to remove the excess of formalin.

- Cryoprotect the tissue sections in a 30% sucrose solution in PBS (pH 7.4) with 0.1% Azide at 4 °C until lung samples sank (2-4 weeks approximately). This solution cryoprotects and prevents microbial contamination.

- Cut lung sections with a cryostat (5 µm-thick sections), placing them in Vectabond-treated glass slides (Vector® Laboratories Ltd., Burlingame, CA) for proper adherence of the tissue sections.

For paraffin-embedded sections:

- Treat formalin-fixed tissue sections with chromic acid to fix the lipids to the tissues before paraffin-embedding, following the protocol described by Tracy & Walia (2002) in detail.

Note: Chromic acid is a very hazardous and possibly carcinogenic chemical (National Center for Biotechnology Information, 2005). Thus, hand it carefully, and always work under a chemical hood.

Oil Red O staining technique:

Oil Red O solution preparation:

Oil Red O stock solution: 100 ml of Isopropyl alcohol (1L, Merck Millipore (ref #109634)) and 0.5 g of oil-red O (Sigma-Aldrich (ref#O0625)). Mix for hours and let stand for 24 hours prior use.

It should be stored in a tightly sealed bottle to prevent solvent evaporation and the consequent Oil Red O precipitation, and covered with aluminum foil, to avoid light exposure, as the stain is photosensitive. This solution is stable for one year.

Oil Red O working solution: 60 ml stock solution and 30 ml of distilled water. Mix and filtrate. Use within the next two hours.

Procedure:

1) Immerse the tissue slides in 60% isopropyl alcohol (prepare with distilled water) for 2 min.
2) Immerse tissue slides in the working solution for 30 min in a dark environment and cover the Coplin staining jar with aluminum foil.
3) Briefly immerse (1 min) the slides in 60% isopropyl alcohol (Merck Millipore, MA, EEUU).
4) Wash the slides for a couple of min in distilled water.
5) Counterstain with a previously prepared Mayer's hematoxylin for 10 min.
6) Wash under tap water for 10 min.
7) Mount the slides in an aqueous mounting medium.

Results:

Neutral lipids appear in red. The background color is from blue to purple when previously fixing lipids with chromic acid, and from light to dark blue when working with frozen sections.

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7.2. OsO_4 PROTOCOL FOR FAT EMBOLISM DEMONSTRATION IN PARAFFIN-EMBEDDED TISSUES

The protocol proposed here for a histological demonstration of lipid material using OsO_4 fixation is an update of the protocol developed by Abramowsky et al. (1981).

General:

Before use, pre-rinse the OsO_4 ampoule and all the laboratory material (bottles, bowl, tweezers) that will contact OsO_4 with nitric acid to prevent contamination. Later, rinse in a few changes of distilled water.

OsO_4 is extremely toxic, as its vapors attack the respiratory system and eyes. It is listed by the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) as fatal if inhaled, swallowed, or in contact with skin (United Nations, 2017). Hand it carefully, and always work under a chemical hood.

Procedure:

1) Trim formalin-fixed lung tissue/s into samples 1 mm thick to ensure an adequate reagent penetration. Place the samples into tissue processing cassettes adequately identified.
2) Dilute the OsO_4 (1g ampoule, Aname® (ref #19100)) in distilled water to get a 1% OsO_4 aqueous solution. Use a 250 ml borosilicate glass bottle or equivalent.

   The process to dilute OsO_4 crystals:

   Under a chemical hood, open the ampoule very carefully and introduce it in the distilled water. In our experience, many crystals remain within the ampoule and not in contact with the water. Pipette several times distilled water inside the ampoule to take out the crystals from the ampoule.

Sonicate the solution in periods of 10 min with 2 hours break. When sonicating, place freezer blocks in contact with the bottle containing the solution to prevent it from warning. During the breaks, keep the solution hermetically sealed in the fridge. Repeat the process until there are no crystals in the solution (usually six sonic passes).

3) Wash the sample/s for 20 min in tap water (use the stainless-steel bowl), followed by 10 min in distilled water.
4) Place the lung sample/s in the OsO_4 solution within a borosilicate glass bottle hermetically sealed and covered with aluminum foil with continuous agitation for approximately two hours. Later, take out the samples using tweezers.
5) Rinse the sample/s in running tap water for 30 min.
6) Prepare a 1% periodic acid (25 g, Aname® (ref #19325)) solution (in distilled water). Prepare just before using it. Use a borosilicate glass bottle.
7) Place the sample/s in the 1% periodic acid solution in agitation until the dark osmicated tissue is uniformly cleared (approximately 30 min).
8) Rinse the sample/s in running tap water for 30 min.
9) Rinse the sample/s in several changes of distilled water.
10) Process and embed the sample/s as usual in paraffin.
11) Cut paraffin sections into slides five-μm thick in the microtome.
12) Introduce the previously dewaxed slide/s into a 1% picric acid (100 g, Aname® (ref #19550)) solution, prepared with 96% ethanol, for 24 hours. This step allows the elimination of diffuse background color in our slide. Use a Coplin staining jar.
13) Rinse the sample/s in running tap water until there is no yellow pigment present in the slides.
14) Rinse the slide/s in several changes of distilled water
15) Counterstain with HE.

Results:

Unsaturated lipids in black and background in pink.

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7.3. LIPID ANALYSIS

Folch procedure for lipid extraction of lung samples:

This protocol has been modified from S. Iverson by H. N. Koopman for blubber/acoustic fat samples and has been later adapted to lung samples.

General:

Use only glass and Teflon materials, as the solvents used in this protocol react with plastic.

Wash glassware (let stand in water and soap overnight), rinse with distilled water and let dry. Before use, pre-rinse all glassware with solvent: methylene chloride ($MeCl_2$).

All solvent work must take place in the fume hood.

Procedure:

1) Weigh ~ 1 g of lung tissue into a 40 ml centrifuge glass tube. Do not take the subsample right at the surface but from the center. Clean the cutting board between samples to avoid cross-contamination.
2) Add 18 mL 2:1 chloroform: methanol with 0.01% BHT.
3) Let sit within the solvent for five days.
4) Mash the lung tissue with a glass rod to release all lipids into the solvent. Rinse rod with hexane between samples.
5) Filter through a plain filter into clean tubes to get rid of lung remains.
6) Add 3 mL MeOH + 3 mL 0.9% NaCl and vortex.
7) Centrifuge 5 min at 2000 rpm.
8) Collect the lower phase (chloroform + lipid). For that, remove the upper phase carefully (without disturbing the interface) and throw it away.
9) Collect the lower phase using a new pipette for each sample and, with a pipet bulb, almost squeezed out, insert pipet tip through the interface

and gently blow out a stream of bubbles from the pipette, so none of the interface gets into the tip of the pipette. Place the pipette tip at the tube's bottom and withdraw the lower phase up to the interface (without including it).

10) Pipette the lower phase into a new glass tube that has a sprinkle of anhydrous Na_2SO_4 in it. Cap, vortex, and let settle.
11) Carefully pipette out the lower phase (avoiding all Na_2SO_4) from the tube into a clean, pre-weighed glass tube.
12) Evaporate under a stream of nitrogen. Weigh, and resuspend in hexane at 50 mg lipid/mL hexane (15 mg/ml in hexane for Iatroscan analysis). Transfer to storage vial and place it in the freezer.

Transesterification (creation of FABEs for GC analysis)

Procedure:

1) The goal is to esterify ~50 mg of total lipid in a total of 1 ml of hexane. Place 50 mg lipid in heavy-duty 10 ml test tube (e.g., Kimax graduated tubes). If total lipid is 50 mg/ml, take 1 ml of the lipid/hexane mixture.
2) Add 1 mL BF3 in butanol.
3) Flush with nitrogen, cap, and vortex.
4) Place in the heating block for 1 hour at 100 ºC.
5) Remove tubes from the heating block and let them cool (10-15 min).
6) Add 3 ml distilled water, vortex.
7) Centrifuge 5 min at 2000 rpm.
8) This time, the lipid is in the upper phase (hexane and lipid). Remove the upper phase to a new, clean tube with a small sprinkle of Na_2SO_4. Cap, vortex, let settle.
9) Transfer hexane/lipid to GC vial with a new pipette, taking care not to put any Na_2SO_4 into the GC vial. Make sure GC vial is appropriately labeled. Wrap lid in DuraSeal and place in the freezer.

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Tesis doctoral

Marina Arregui Gil

Fat embolism in stranded cetaceans
(Embolismo graso en cetáceos varados)

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