LUNG FUNCTION, RESPIRATORY DISEASE, AND ENERGETIC REQUIREMENTS OF MARINE MAMMALS: IMPLICATIONS FOR CONSERVATION

• DOCTORAL THESIS •
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VNIVERSITAT DE VALÈNCIA

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Striped dolphin, *Stenella coeruleoalba* (Meyen, 1833)
Garraf, Mediterranean Sea, 2018
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VNIVERSITAT DE VALÈNCIA
Facultat de Ciències Biològiques
Departament de Zoologia

ROMANA CAPACCIONI AZZATI, Profesora Titular del Departamento de Zoología de la Facultat de Ciències Biològiques de la Universitat de València, y ANDREAS FAHLMAN, Investigador de la Fundación Oceanogràfic de la Comunitat Valenciana, como codirectores de la Tesis Doctoral que se detalla a continuación:

CERTIFICAN:

Que Dª. ALICIA BORQUE ESPINOSA ha realizado bajo su dirección y con el mayor aprovechamiento el trabajo de investigación recogido en esta memoria y que lleva por título:

"LUNG FUNTION, RESPIRATORY DISEASE, AND ENERGETIC REQUIREMENTS OF MARINE MAMMALS: IMPLICATIONS FOR CONSERVATION"

para optar al grado de Doctora por la Universitat de València.

Y para que así conste, en cumplimiento de la legislación vigente, expedimos el presente certificado en Valencia, a 7 de octubre de dos mil veintiuno.

ROMANA JULIANA\|CAPACCIONI\|AZZATI

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“The sea, once it casts its spell, holds one in its net of wonder forever.”

- Jacques Cousteau -

“It is the worst of times, but it is the best of times because we still have a chance.”

- Sylvia Earle -

Dedicatoria
Dedication

Bottlenose dolphin, *Tursiops truncatus* (Montagu, 1821)
Red Sea, 2019
Photo credits: Sergio Rubio Gracia

A mi familia:

A mi querido abuelito, a quien llevo tantos años echando de menos,

A mi querida abuelita que se marchó en el ecuador de este trabajo con 102,

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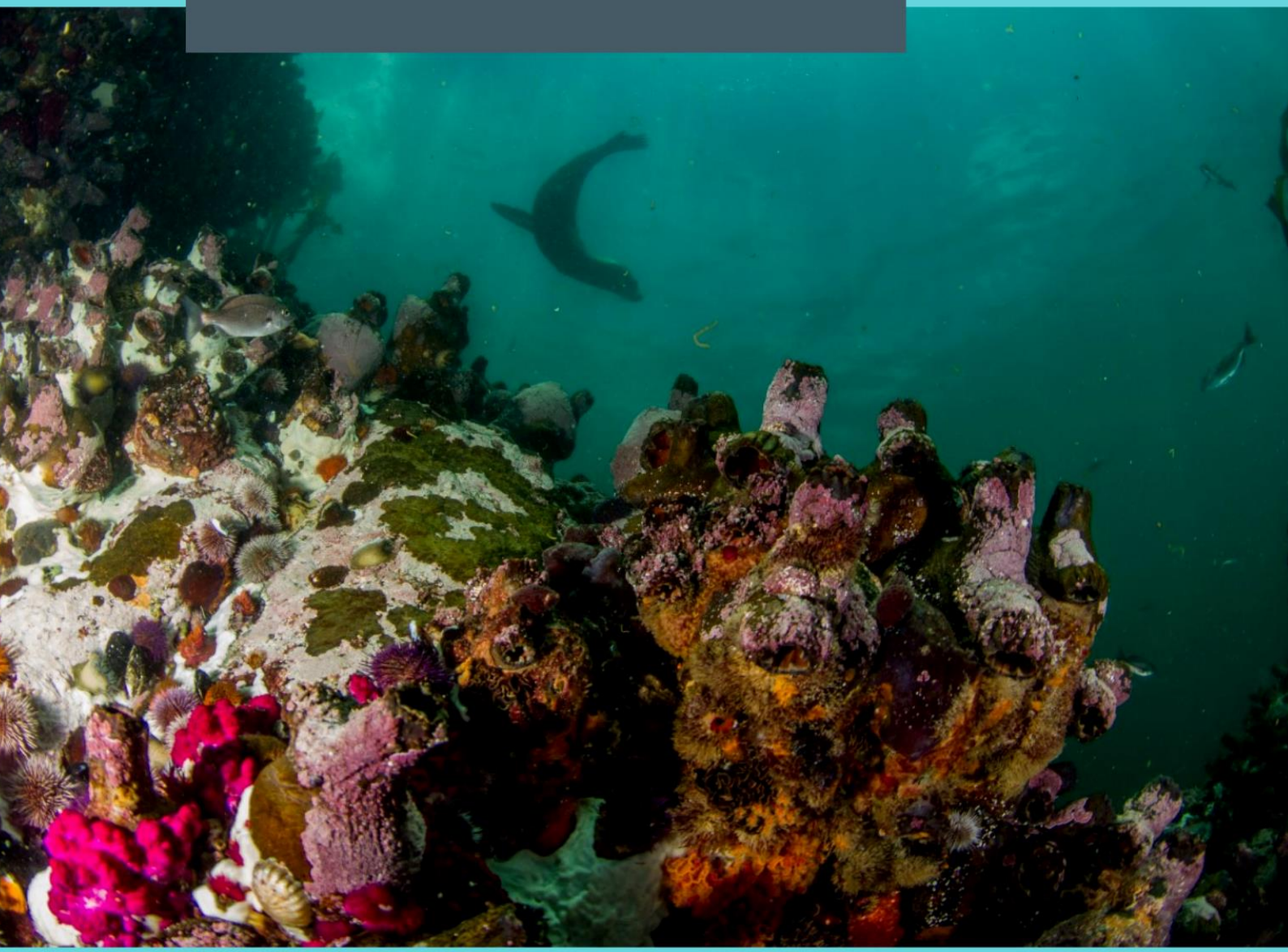

Cape fur seal, *Arctocephalus pusillus pusillus* (Schreber, 1776)
Cape town, Atlantic Ocean, 2016
Photo credits: Vicente Renovell Farré

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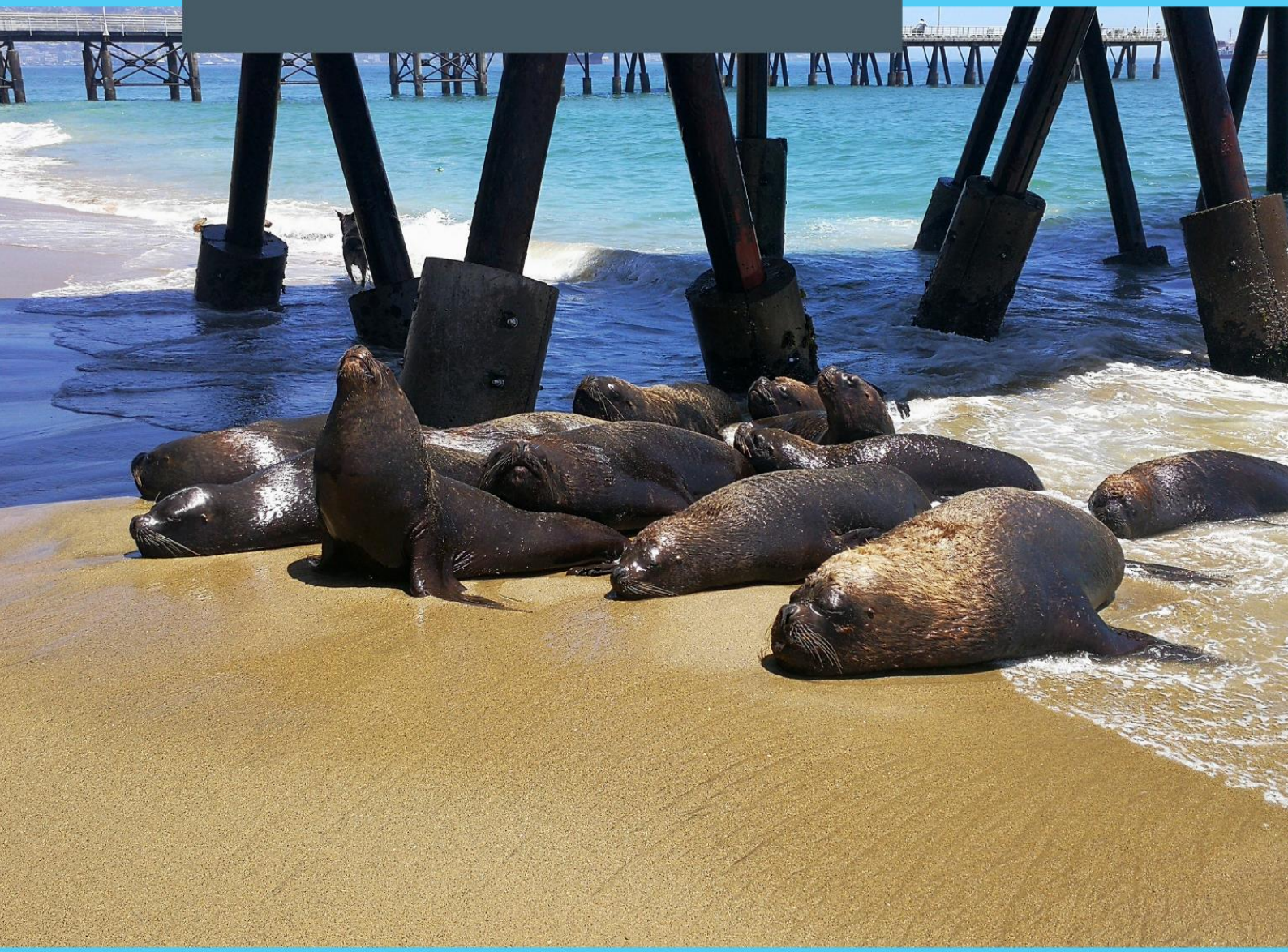
Table of contents



South American sea lion, *Otaria byronia* (Blainville, 1820)
Valparaíso, Pacific coast, 2018
Photo credits: Antonio Borque Espinosa

Table of contents

Abstract

Common dolphin, *Delphinus delphis*, Linnaeus, 1758
Bosphorus strait, 2021
Photo credits: Sara Sánchez Quiñones

Abstract

Marine mammals rely on their diving capacity to survive as this determines their foraging efficiency. Ultimately, their anatomy and physiological capacity, and the limitations imposed by the environment determine the maximal time underwater. Thus, the behavioral decisions undertaken by marine mammals would be related with the different combination of these factors. As air breathing mammals, respiratory function may alter the diving capacities and limit foraging. Further, respiratory disease has been reported as one of the major causes of mortality in these species and may have consequences for their respiratory function and diving capacities. In addition, the ability of marine mammals to efficiently use the available O_2 stores during diving has been recognized as an important component that determines diving behavior and the duration of the breath-hold. However, the increasing human activity in the marine ecosystem (e.g., climate change, overfishing, contamination, etc.) is resulting in environmental alterations that could force marine mammals to perform dives beyond their physiological scope. In addition, these perturbations may disrupt the normal physiological mechanisms that enhance diving, or may also increase their susceptibility to disease, which could have consequences on their diving capacity. Therefore, a better understanding of species-specific normal respiratory function and capacity could help improve our understanding about respiratory limitations and the functional consequences of respiratory disease, and how these affect the diving capabilities of marine mammals. In addition, studies evaluating the metabolic rate, or O_2 consumption rate, during diving in different species will help understand the energetic requirements of underwater behaviors, such as foraging or travelling. This information could help understand interspecific limitations that drive behavioral decisions within a changing environment. Such information would be vital to enhance conservation efforts of those populations suffering increased direct or indirect impacts that could affect their future survival.

The present thesis is aimed to provide basic information about respiratory function and energetic requirements during in-water activities in one of the species facing an increased habitat loss: the Pacific walrus (*Odobenus rosmarus divergens*). In addition, the present investigation assessed the use of spirometry as a non-invasive diagnostic tool to evaluate respiratory health and the functional consequences of

respiratory disease in the bottlenose dolphin (*Tursiops truncatus*). These objectives were performed with the participation of trained animals housed in professional care and were developed in three independent chapters briefly summarized below:

In Chapter I, basic respiratory function was measured in three adult Pacific walruses. The results showed an enhanced ventilatory capacity (e.g., higher tidal volume and lung compliance, and high respiratory flows maintained over the entire respiratory manoeuvre) compared with terrestrial mammals. Respiratory function was assessed in different body positions on land and in water, which showed flow limitations while lying on land and increased respiratory flows when in water. These results provide basic information to understand respiratory limitations in this species, and suggest that respiratory function in semi-aquatic marine mammals should be evaluated both on land and in water.

In Chapter II, lung function testing (spirometry) was used as a non-invasive method to assess respiratory health in three adult bottlenose dolphins. Lung health was assessed through the development of lung function indices adapted for the respiratory capacity of dolphins, and the evaluation of the flow-volume relationship. The results were compared with clinical diagnostics (e.g., blood and sputum samples) and chest radiographs, and showed that lung function testing could detect respiratory disease, evaluate functional changes, and assess treatment efficacy in dolphins trained to exhale maximally while in water. In addition, the results indicated that spirometry could provide diagnostic information in stranded individuals while breathing spontaneously. Thus, the results presented in this chapter showed that lung function testing is a potential non-invasive method to evaluate respiratory health in trained and stranded dolphins.

In Chapter III, the O_2 consumption rate was measured in three adult Pacific walruses while floating at the water surface, and during short and shallow stationary dives and subsurface swimming. The results are similar to previously results reported for adult pinnipeds where measured metabolic rates during inactive periods (on land or in water) were greater than those expected from similarly sized terrestrial mammals. In addition, metabolic rate during diving was lower as compared with periods at the surface, as previously reported for other pinniped species. This shows that the walrus has behavioural or physiological means to limit metabolic costs during diving. The data presented in this chapter could help improve previous bioenergetic models aimed to quantify the consequences of environmental change in the Pacific walrus.

List of figures and tables

Galapagos sea lion, *Zalophus wollebaeki* (Sivertsen, 1953)
Galapagos, Pacific coast, 2014
Photo credits: Julio Sanjuan

List of figures and tables

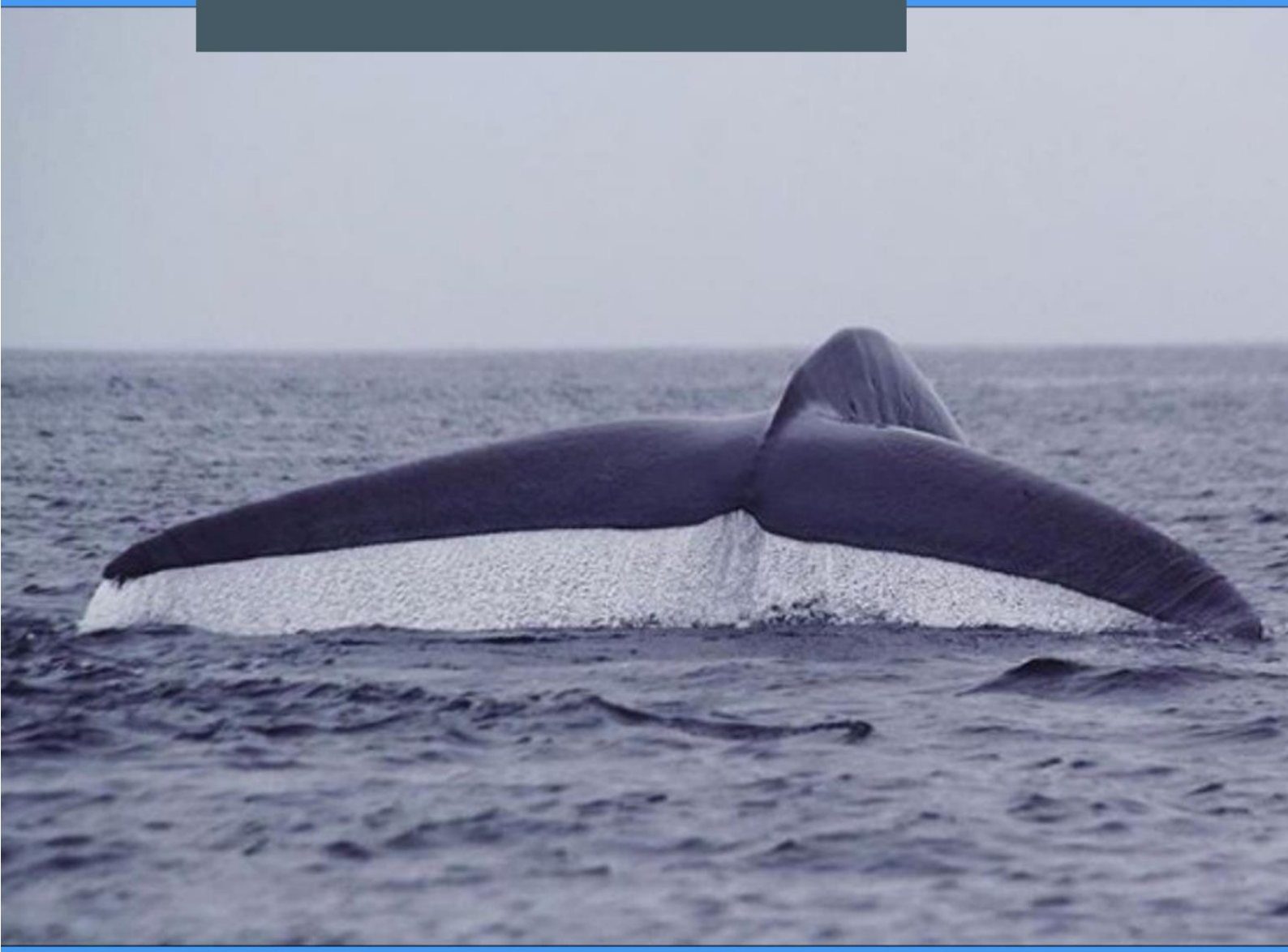
Figures

Appendix

Tables

Supplementary tables

1. General introduction



Blue whale, *Balaenoptera musculus* (Linnaeus, 1758)
Chile, Pacific Ocean, 2017
Photo credits: Clara Agusti Pujol

1. General introduction

1.1. Marine mammals: a life beneath the surface

The early terrestrial ancestors of marine mammals were provided with the opportunity to explore and exploit additional resources in the most accessible coastal habitats of the marine ecosystem. The increasing incursions into the ocean, together with the evolutionary mechanisms, assisted on the progressive development of anatomical and physiological adaptations that allowed this group of mammals to successfully occupy the marine environment (Berta et al., 2006; Castellini and Mellish, 2015; Reidenberg, 2007). These adaptations allowed marine mammals to deal with the inherent extreme environmental conditions of this aquatic environment, such us increased water density compared to atmospheric air, increasing hydrostatic pressure with depth, absence of light, or the high specific heat capacity of water. However, one of the major restrictions that these air-breathing animals overcame was the separation of the nutritional marine resources from the other vital resource for living: the O_2. Thus, the terrestrial ancestors of marine mammals had to acquire specific adaptations to efficiently use the O_2 inspired at the surface while diving for food. The different breath-hold needs, derived from the diverse foraging opportunities at the marine environment, promoted the development of different diving capabilities. Consequently, the evolutionary history of marine mammals led to a widely diversified group of species that have occupied several marine ecological niches (Berta et al., 2006).

Nowadays, this group of mammals completely rely on marine resources to survive. While the majority of living species spend most of their time underwater displaying a great variety of behaviors (e.g., social, foraging, exploratory, reproductive, etc.), they are classified as semi-aquatic or fully-aquatic species depending on the degree of which they spend part of their life on land. Whether they belong to the first or the second group, marine mammals show a wide range of diving capabilities in terms of dive duration, depth, and frequency of underwater behaviors, which differ among species and even within taxonomical groups (Ponganis, 2011; Ponganis et al., 2003). This variability, and the extreme characteristics of observed diving behaviors in some species, such us dives of up to 3.5 hours and to depths exceeding 3000 meters in beaked whales (Quick et al., 2020), raised several questions about the underlying physiological mechanisms that would allow these extreme diving behaviors (Castellini, 2012). It was early suggested that

diving mammals have physiological adaptations that would enhance their O_2 storage capacity and reduce the O_2 utilization while diving. This working hypothesis was first developed by studies initiated by Laurence Irving and Per Scholander in the 1940s. The results from these studies linked cardiorespiratory anatomy and physiological function, which they proposed would explain the diving capacities in marine mammals. These first investigations helped develop a framework that would provide the basis for subsequent research efforts in the field of diving physiology. Since then, scientists have continued investigating the diverse underlying mechanisms and adaptations that allowed marine mammals to survive beneath the surface (For generalized literature see: Berta et al., 2006; Castellini and Mellish, 2015; Kooyman, 1989; Perrin et al., 2009; Ponganis, 2015). However, to focus on the scope of the present thesis, the following sections describe the initial and subsequent findings that highlighted the relevance of respiratory function and O_2 management for the ability of marine mammals to successfully exploit the marine environment.

1.2. Respiratory system: gas exchange and pressure at depth

Respiratory anatomy: towards an efficient ventilatory capacity

During diving, marine mammals consume the limited amount of inspired O_2 at the surface while producing the subproduct derived from cellular aerobic metabolism, the CO_2. During the recovery between dives at the surface, the respiratory system provides access to replenish the O_2 in the circulatory system while eliminating the accumulated CO_2. The limited amount of available O_2 during the dive was suggested to be related with the accumulated O_2 in the blood, lungs, and tissues (Scholander, 1940). Thus, the respiratory function and capacity were early considered relevant components to be investigated in order to understand the diving capabilities in marine mammals. During the first investigations on respiratory function in marine mammals, Per Scholander (1940) documented reinforced conducting airways associated with high lung and chest compliance in some species of cetaceans and seals. These initial anatomical observations led him to suggest that the flexible chest in these animals would tolerate compression, decreasing the differences between intrathoracic and environmental underwater pressures at depth and helping avoid lung squeeze during diving. In addition, Scholander suggested that the air contained in the compliant lungs could be displaced into the less compressible conducting airways with increasing hydrostatic pressures, allowing for alveolar collapse

at depth. The alveolar collapse would gradually decrease the gas diffusion rate between blood and lungs with increasing diving depths, causing a pulmonary shunt that would eventually result in cessation of gas exchange. This reduction in gas exchange at depth would help avoid N_2 accumulation during the dive, reducing the risk of inert gas narcosis, and the potential for gas-bubble formation that causes vascular embolism and decompression sickness (DCS).

Later studies using both, excised tissues and living animals, confirmed that the reinforced conducting airways and high lung and chest compliance were also observed in other species of marine mammals (Denison and Kooyman, 1973; Denison et al., 1971; Fahlman et al., 2011; Fahlman et al., 2014; Fahlman et al., 2015; Kooyman, 1973; Kooyman and Sinnett, 1979; Leith, 1976; Olsen et al., 1969; Piscitelli et al., 2010; Ridgway et al., 1969; Tarasoff and Kooyman, 1973). Further anatomical investigations have shown that there is significant variation in the structural properties of the conducting airways, which are related to differences in the relative distribution of cartilage or muscle in the upper and/or lower respiratory airways (Bagnoli et al., 2011; Cozzi et al., 2005; Davenport et al., 2013; Henk and Haldiman, 1990; Moore et al., 2014; Piscitelli et al., 2010; Piscitelli et al., 2013). In addition, together with these functional and anatomical investigations, a few studies confirmed Scholander's hypothesis about alveolar collapse by determining the effect of pressure on gas exchange (Kooyman et al., 1972; Kooyman and Sinnett, 1982; McDonald and Ponganis, 2012), or even reporting chest and respiratory pressure-related compression in postmortem animals under hyperbaric conditions (Denk et al., 2020; Moore et al., 2011).

However, besides the relevance of the reinforced conducting airways and the high chest and lung compliance for avoiding barotrauma during deep diving, Denison et al. (1971) additionally suggested that the respiratory anatomy may also have consequences on respiratory function. It was suggested that the reinforced conducting airways may help maintain the airways fully expanded while generating high respiratory flows ($\dot{V}$), unlike the flexible distal airways in humans that compress while emptying the lungs which limit the maximum breathing capacity (Denison et al., 1971). In order to evaluate the functional consequences of these initial anatomical findings, the most frequent anatomical studies were followed by an increased effort to measure basic respiratory function in live animals. Some of these studies helped confirm that marine mammals are able to generate higher $\dot{V}$ during breaths of short duration as compared with terrestrial mammals (for examples see

Table 1 in Fahlman et al., 2017). Other studies that evaluated the relationship between $\dot{V}$ and volume during maximal and spontaneous respiratory manoeuvres, showed that this high $\dot{V}$ is maintained almost constant even at low lung volumes while emptying the lungs (Fahlman et al., 2019; Fahlman et al., 2015; Kerem et al., 1975; Kooyman and Cornell, 1981; Kooyman et al., 1975; Kooyman and Sinnett, 1979; Leith et al., 1972; Matthews, 1977; Olsen et al., 1969). Thus, it is likely that the characteristic airway reinforcement in marine mammals allows high $\dot{V}$ that are frequently maintained over the entire respiratory manoeuvre. In addition, it has been suggested that the high chest compliance would allow for an increased elastic recoil of the lungs during the expiration (Fahlman et al., 2017), which explains how these animals are capable of almost emptying their lungs in a single breath (Denison et al., 1971; Fahlman et al., 2011; Fahlman et al., 2015; Irving et al., 1941a; Kooyman and Cornell, 1981; Kooyman and Sinnett, 1979; Olsen et al., 1969; Scholander, 1940).

However, when comparing $\dot{V}$ and breath duration among species, there appears to be considerable variability (Fahlman et al., 2017; Ponganis, 2011), possibly related to the reported respiratory anatomical differences between certain species. In fact, this variation in respiratory anatomy has also been proposed as an adaptation to the diverse diving behaviour and life history within species (Denison and Kooyman, 1973; Fahlman et al., 2017; Moore et al., 2014). For example, some otariids and cetaceans are considered shallow divers that recover from consecutive dives through rapid respirations while swimming (Denison and Kooyman, 1973; Piscitelli et al., 2013). These species would therefore benefit from stiffer conducting airways that allow a higher $\dot{V}$, which would reduce recovery time through rapid respiratory manoeuvres while short surfacing bouts during swimming (Denison and Kooyman, 1973; Piscitelli et al., 2013; Ponganis, 2011). Experimental evidence has shown that the unique architecture of the respiratory system in marine mammals has relevant consequences that help improve the volume of exchanged air during respiration, or the ventilatory capacity. These anatomical properties would help maximize gas exchange during surface intervals allowing continuous swimming and faster recovery following a long dive (Fahlman et al., 2017; Ponganis, 2011). Nevertheless, the functional properties of the respiratory system have also direct consequences on the characteristics of ventilatory volumes in marine mammals.

Efficient ventilatory capacity: consequences for lung volumes and breathing frequency

Studies have shown that marine mammals are able to exchange up to 90% of their total lung capacity (TLC) in a single breath (Fahlman et al., 2015; Irving et al., 1941a; Kooyman and Cornell, 1981; Olsen et al., 1969). Thus, some species have reported to have a vital capacity (VC) close to TLC and a small residual volume (Fahlman et al., 2011; Kooyman et al., 1973; Piscitelli et al., 2010). This would help reduce dead space and improve gas exchange (Fahlman et al., 2017). In addition, the tidal volume (V_T), is generally higher in marine mammals (for examples see Table 1 in Fahlman et al., 2017) as compared with similarly sized terrestrial mammals (Stahl, 1967). Nevertheless, it is possible that this large exchange capacity previously described represents the upper capacity of marine mammals, as measured V_T has been frequently reported to be lower than VC in resting animals or even following exercise (Fahlman et al., 2019; Fahlman et al., 2015; Fahlman and Madigan, 2016; Fahlman et al., 2020a; Irving et al., 1941a; Kooyman and Cornell, 1981; Olsen et al., 1969; Reed et al., 2000). Still, the increased reported V_T as compared with their terrestrial counterparts may have facilitated the reduction of breathing frequency (f_R) in these aquatic animals (Mortola and Limoges, 2006), while allowing to maintain the same minute ventilation (Fahlman et al., 2017; Mortola and Sequin, 2009). Collected data on V_T and f_R have also showed a high interspecific variability (Fahlman et al., 2017). However, these respiratory variables could be modified depending on the gas exchange needs and the consequently respiratory effort. Therefore, the diverse circumstances during such measurements (e.g., exercise needs) could explain the reported differences between species (Fahlman et al., 2017).

On the other hand, TLC in marine mammals has been reported to be similar to that described for terrestrial mammals (Fahlman et al., 2011; Kooyman, 1973; Piscitelli et al., 2013; Ponganis, 2011; Ponganis, 2015). While an increased lung size would be an efficient solution to increase the quantity of O_2 that could be distributed in the body during the dive, the fact that marine mammals appear to possess similar TLC than that reported for their terrestrial counterparts has been suggested to be beneficial for reducing the buoyancy forces and the N_2 uptake during diving (Piscitelli et al., 2010). The O_2 storage during diving is mainly distributed in three compartments: the respiratory system, blood, and muscle (Costa, 2007; Ponganis, 2011). However, most of marine mammals primarily rely on the blood and muscle O_2 storage capacity, which derive from the larger blood volume and the greater concentrations of blood haemoglobin and muscle myoglobin as

compared to terrestrial mammals (Costa, 2007; Ponganis, 2011). Nevertheless, the initial amount of available O_2 that will be utilized during the dive relies on the lung O_2 concentration at the beginning of the breath-hold, which is related with the exchange capacity of the respiratory system.

The results from almost one century of investigations have provided insights about how the respiratory system works to provide an enhanced exchange capacity and to overcame pressure-associated problems. While pressure-associated constrains during diving may have imposed a limitation for larger lung sizes in marine mammals, the maximized exchange capacity and the consequent large volumes displaced during respiration, allowed the respiratory system to become an enhanced compartment to load and distribute the O_2 that will be utilized during the dive. Nevertheless, marine mammals possess an additional suite of physiological mechanisms that operate to maximize the utilization of the available O_2 provided by the respiratory system.

1.3. Dive response and oxygen utilization

Bradycardia, selective blood perfusion and reduced oxygen utilization: the dive response

The aerobic metabolism during breath-hold consumes the limited amount of available O_2 while producing and accumulating CO_2, and extended periods of breath-hold leads to hypoxia and hypercapnia. Therefore, the limited amount of exchanged O_2 in the lungs, blood, and muscle, should be carefully utilized to supply aerobic activity while exercising during diving. Laurence Irving and Per Scholander early realized that despite the increased O_2 stores in marine mammals as compared with their terrestrial counterparts, these would not be sufficient to maintain the aerobic activity during their extended dives if the O_2 consumption (or metabolic rate) was similar to that measured at the surface (Irving, 1934; Irving, 1939; Scholander, 1940). Irving suggested that an alternative solution could be to incur an O_2 debt that would be re-paid after surfacing (Irving, 1934). The energetic supply during the hypoxic underwater periods would be managed by the anaerobic pathway with the consequent production of lactic acid, one of the metabolites produced during anaerobic metabolism (Irving, 1934). However, Irving highlighted that this alternative could only be useful for a limited number of dives due to the tissue-associated toxicity of lactate (Irving, 1934; Irving, 1939). Based on earlier observations in diving ducks that had revealed a decreased heart rate during forced dives, Irving suggested that marine mammals may have acquired similar cardiovascular

mechanisms that, under neural control, would allow the body to balance the aerobic metabolism and O_2 availability during diving (Irving, 1934; Irving, 1939).

The cardiovascular changes observed in other diving species during breath-hold proceeded with forced diving laboratory studies to evaluate the cardiovascular effects of breath-holding in marine mammals. During these early investigations, Irving and Scholander documented a decreased heart rate (or bradycardia), a decrease on the rate of arterial O_2 depletion, and a post-dive increase in blood lactate concentrations (Irving et al., 1941b; Irving et al., 1942; Scholander, 1940). Based on these results, it was proposed that during diving, marine mammals experience peripheral vasoconstriction, where the blood flow is conserved for vital organs that have sustained O_2 requirements such us brain, lungs, and heart. Peripheral and non-vital organs, on the other hand, only receive limited O_2 and would rely on anaerobic metabolism during periods of O_2 isolation, resulting in increasing post-dive blood lactate concentrations (Irving et al., 1941b; Irving et al., 1942; Scholander, 1940). Both, bradycardia and peripheral vasoconstriction, would decrease the cardiac output, hence the work of the heart and its O_2 consumption (Irving et al., 1941b; Scholander, 1940). In addition, the peripheral vasoconstriction would result in less tissues being perfused, with an associated drop in blood and lung O_2 depletion (Irving et al., 1941b; Scholander, 1940). Consequently, this cardiovascular regulation was suggested to contribute to the reduction of O_2 delivered and consumed in the tissues, which would help explain the reported decrease in O_2 consumption during breath-hold below resting levels (Irving et al., 1941b; Scholander, 1940).

These first investigations provided evidence to confirm the initial hypotheses that marine mammals have physiological adaptations to help conserve O_2 during diving and maximize the time spent underwater. The pioneering work done by Irving and Scholander's set the basis for the work in diving physiology for the following 80 years, where investigations were aimed to confirm these initial findings in other species of marine mammals, and to further explore other suggested adaptations to breath-hold such us O_2 storage capacity in blood and muscle, or biochemical adaptations to tolerate hypoxia, hypercapnia, or increased blood lactate levels (for reviews see: Castellini, 2012; Ponganis, 2011). In regards to the use of O_2 during diving, further studies supported depressed metabolism during breath hold by observing decreased body temperature, or by measuring the reduction in the blood flow to certain tissues or the cessation of determined physiological activities such us digestion or urine formation (for reviews see

Castellini 2012). The cardiovascular response, decreased metabolic rates (or hypometabolism), and enhanced O_2 stores, were later unified under the concept of "diving reflex" and shown to exist in all air-breathing species (Scholander, 1963). As technology advanced, the use of restrained animals progressively moved towards data collection using data-logging devices on free-diving or trained animals, which showed that the cardiovascular response was less intense during voluntary breath-holds (Bartholomew, 1954; Elsner, 1965; Elsner et al., 1966). An important breakthrough in this field was provided in further studies showing that this cardiovascular response is not a reflex as initially suggested, but may instead be a modulated response depending on the specific diving requirements (for examples see: Andrews et al., 1997; Fahlman et al., 2020b; Fedak et al., 1988; Kooyman and Campbell, 1972; Ridgway et al., 1975; Thompson and Fedak, 1993).

However, besides the intensified study of the cardiovascular changes operating during diving, development of new technologies and tracking devices from the 1980s, permitted an explosion in studies investigating dive behavior, and numerous studies began to define the normal dive duration and depth of several species of marine mammals (for reviews see: Castellini, 2012; Ponganis, 2011). This bio-logging technology showed that, although these animals possess the capacity to perform extremely long and deep dives, most breath-holds are below their reported extreme records (for reviews see: Butler and Jones, 1997; Castellini, 2012). These findings suggested that marine mammals mostly rely on aerobic metabolism while avoiding anaerobic pathways. One early study comparing the blood lactate accumulation with dive frequency and duration in free diving Weddell seals, reported that blood lactate concentrations increased during the less frequent longer dives (~20 min, Kooyman et al., 1980). Based on these results, the term aerobic dive limit (ADL) was formulated, which was defined as the extent to which a breath-hold animal could prolong the dive while avoiding an increase in blood lactate accumulation (Kooyman et al., 1980). These observations in the Weddell seals provided evidence that most dives in marine mammals may be short and aerobic in nature, and that these animals would only dive beyond their ADL on occasion (Kooyman et al., 1980). By diving on aerobic metabolism, marine mammals reduce the longer surface intervals required to eliminate the accumulated by-products from anaerobic metabolism for those dives exceeding the ADL (Kooyman et al., 1983). Since direct measurements of blood lactate accumulation are inherently challenging, the ADL has been frequently estimated

(calculated ADL: cADL) using the calculated total O_2 stores and the O_2 consumption rate during diving or diving metabolic rate (DMR) (Butler and Jones, 1997). Kooyman's hypothesis shifted the interest towards understanding how the dive response affected DMR. Thus, some of the subsequent studies in the field measured the DMR to provide estimations of the cADL that could be compared with behavioral observations. The energetic investment during diving is an important component of the total energy budget in marine mammals. Therefore, DMR and cADL estimations have provided relevant information to better understand the energetic management and the metabolic conditions that underlie diving behavior in marine mammals.

Oxygen utilization and energetic requirements during diving

The inherent challenges of measuring energetic expenditure in free-ranging animals have limited the possibilities of obtaining direct measurements of DMR in the wild. Most of the work performed with free-ranging marine mammals has used stables isotopes or indirect proxies (e.g., heart rate and f_R) to estimate or predict the field metabolic rate (FMR) (for examples see Butler and Jones, 1997). However, these proxies include measurements over several days where the animals could display a variety of different activities (e.g., exploring or foraging dives, travelling, resting, etc.,). Only a few studies have obtained direct measurements of DMR on a dive-by-dive basis or during a diving bout, where most of the work has been performed in trained pinnipeds (Fahlman et al., 2008a; Fahlman et al., 2013; Hastie et al., 2007; Hurley and Costa, 2001; Sparling and Fedak, 2004) and measures on free-diving animals are scarcer (Castellini et al., 1992; Kooyman et al., 1973). DMR has been estimated either as the O_2 consumed during the post-dive recovery in excess of the resting levels, or as the O_2 consumed during the post-dive recovery divided by the dive cycle (dive duration + post-dive duration). Previous studies have showed that DMR during dives of long duration is usually below or close to metabolic rates measured while resting for both, inactive (Castellini et al., 1992; Hastie et al., 2007; Hurley and Costa, 2001) and active dives (Castellini et al., 1992; Fahlman et al., 2008a; Fahlman et al., 2013; Kooyman et al., 1973; Sparling and Fedak, 2004). Furthermore, for those studies where the animals performed dives of different duration, the DMR was inversely proportional to the dive duration (Castellini et al., 1992; Fahlman et al., 2008a; Fahlman et al., 2013; Fahlman et al., 2008b; Reed et al., 1994; Sparling and Fedak, 2004). These results support the early investigations on diving physiology which proposed that DMR is decreased in wild animals (Irving et al., 1941b; Kooyman et al.,

1973; Scholander, 1940). In addition, some of these studies have indicated that the degree of the metabolic response may be highly variable among different species (Castellini et al., 1992; Hurley and Costa, 2001; Kooyman et al., 1973; Reed et al., 1994; Sparling and Fedak, 2004), which has been suggested to be related with the specific swimming strategies or activity level, nutritional state, and foraging behaviours (Hastie et al., 2007; Rosen et al., 2017; Sparling and Fedak, 2004). On the other hand, those studies comparing the cADL and recorded diving behaviour have indicated that some benthic foraging species may regularly exceed their cADL (for reviews see Butler and Jones, 1997). Thus, to better understand the relationship between the diving behaviour and the degree in which marine mammals rely on aerobic metabolism during diving, it is crucial to have accurate estimates of species-specific DMR and their total body O_2 stores.

The experimental evidence in the diving physiology field has shown that marine mammals appear to possess a generalized suite of physiological mechanisms that help prolong their time underwater. While the extent in which these physiological mechanisms operate in different species and behavioral circumstances is still poorly understood, it is recognized that the reduction of the O_2 utilization during a breath-hold is an important component to extend the aerobic breath-hold. However, the knowledge about degree and frequency in which these animals may incur a O_2 debt while relying on the anaerobic metabolic pathway is still rudimentary.

1.4. The relevance of studying respiratory function and energetics for the conservation of marine mammals

Studies on the anatomical and functional properties of the respiratory system in marine mammals have provided a generalized framework to better understand how the respiratory system works to provide adequate ventilation at the surface while preventing pressure-associate problems at depth. These studies have provided information of the functional traits of the respiratory system that help define the limitations of diving. For example, theoretical modelling studies have indicated that respiratory functional characteristics (e.g., compliance) determine the effects of pressure on the respiratory system and the distribution of the air throughout the respiratory airways during diving (Bostrom et al., 2008; Fahlman et al., 2009). Similarly, the investigations aimed to explore the rate in which O_2 is utilized during dives have provided relevant information to better understand the diving capabilities of different species of marine mammals. This

information has been also used to generate predictive models aimed to estimate the energetic expenditure of diving, or the cost of flight responses for animals in the wild (Sparling and Fedak, 2004; Williams et al., 2017). However, although there appears to be a general trend in certain functional characteristics of the respiratory system and the efficient O_2 use during diving among marine mammals, these results have also highlighted differences among species and taxonomical groups that may result in diverse functional and physiological limitations (for reviews see: Butler and Jones, 1997; Fahlman et al., 2017; Piscitelli et al., 2010; Piscitelli et al., 2013). This variation makes it difficult to extrapolate certain basic parameters to those species where there is still limited information. Furthermore, many aspects of respiratory physiology in marine mammals are still unknow (e.g., how pressure affects gas exchange), and our understanding about the energetic requirements and partitioning between aerobic and anaerobic metabolism for different type of dives and species is still rudimentary. Therefore, a better knowledge about basic lung function and energetic requirements would be beneficial to understand the diving capacities of marine mammals.

The survival of marine mammals depends on their diving capacity as this will determine their foraging efficiency. During diving, marine mammals deal with their inherent physiological limitations (e.g., CO_2 and N_2 accumulation, proper management of O_2 consumption, etc.) and the restrictions imposed by the environment (e.g., prey density, pressure at depth, water temperature, etc.), while maximizing foraging success and avoiding potential risks (Hooker et al., 2012). The particular circumstances and combination of these factors would have consequences in the determination of the diving behavior (Hooker et al., 2012). However, the increasing impact of human activity over the last decades (e.g., climate change or over-fishing), is resulting in alterations in the marine environment that could force marine mammals to function beyond their physiological capacity. For example, some studies have reported severe bubble-associated lesions in single- or mass-stranded cetaceans (Cox et al., 2006; Fernandez et al., 2005; Jepson et al., 2003; Jepson et al., 2005), where some of these stranding events were associated with military sonar or seismic surveys (Fernandez et al., 2005; Jepson et al., 2003). Although it has been suggested that marine mammals may be able to deal with the presence of some level of gas emboli (Dennison et al., 2012; Moore and Early, 2004), the exposure to anthropic sources of underwater sound could have altered the diving behaviour of these stranded cetaceans, causing a N_2 accumulation that exceeds their

threshold (Fernandez et al., 2005; Jepson et al., 2003). A recent hypothesis has suggested that marine mammals may be able to manage N_2 accumulation by selectively directing the blood flow through collapsed alveoli while avoiding non collapsed lung regions during the dive (Garcia Párraga et al., 2018). This hypothesis provides an explanation to better understand how underwater disturbances and stress may alter physiology, causing pressure-associated problems (Garcia Párraga et al., 2018). Furthermore, a potential concern that may limit the respiratory physiological scope of marine mammals is respiratory disease. Pulmonary and respiratory tract disease show high prevalence among marine mammals and is considered one of the major causes of mortality in these species (Baker, 1992; Gonzales-Viera et al., 2011; Medway and Schryver, 1973; Sweeney and Ridgway, 1975). While the origin of respiratory disease is well known (e.g., fungal, parasitic, or bacterial: Gonzales-Viera et al., 2011; Sweeney and Ridgway, 1975), the functional consequences have been poorly investigated and may lead to a reduction in both respiratory and diving capacity of marine mammals. The increasing human-made activity and environmental change are resulting in direct (e.g., exposure to contaminants or high temperatures) and indirect (e.g., starvation or decreased water quality) effects that have been reported as relevant factors contributing to disrupt the normal functioning of the immune system in marine mammals, increasing the susceptibility to pathogens and disease (Learmonth et al., 2007; Ross, 2002). In addition, marine mammals may be particularly vulnerable to changes on the availability and distribution of their main prey resources due to environmental change (Learmonth et al., 2007; Simmonds and Isaac, 2007). These potential modifications on access to prey could eventually force these animals to perform longer or deeper dives while diving for food, which could be limited by their physiological scope.

It is likely that potential impacts of the environment could disrupt the normal mechanisms operating during diving in marine mammals, which could result in a departure from the optimal physiological responses in these species. Current projections of anthropic activity and environmental change highlight the necessity of improving our knowledge about the physiological capabilities and limitations in these species. The experimental evidence compiled during the last years has showed that there is no generic marine mammal, and that extrapolations between species should be done with caution. Therefore, the physiological specific differences should be properly evaluated in order to accurately define species-specific capabilities and limitations that would help assess

possible future consequences for the survival of each species. For example, independent studies aimed at establishing species-specific baseline parameters of respiratory function would help improve theoretical models aimed to predict the impact of climate change on the diving capacity of marine mammals. These models accounted for the structural and functional properties of the respiratory system (e.g., compliance or lung volumes) to define the physiological limitations in diving marine mammals (Bostrom et al., 2008; Fahlman et al., 2009; Fahlman et al., 2018; Hodanbosi et al., 2016; Hooker et al., 2009). These baseline respiratory parameters from healthy animals will also be useful to understand the functional consequences of respiratory disease, and to provide novel methodologies to evaluate respiratory health. For example, pulmonary function testing is the standard methodology to assess lung health in humans (Crapo, 1994; García-Río et al., 2013), and could be used for improving veterinarian diagnostics for those animals maintained under human care, and to help evaluate the health status of stranded animals or wild populations (Fahlman et al., 2021; Fahlman et al., 2020a). Understanding the role of respiratory dynamics in wild populations could also help while assessing and monitoring the potential impacts of environmental disasters such as large-scale oil spills (Venn-Watson et al., 2015; Williams et al., 2011). Similarly, further investigations aimed at elucidating the degree in which the O_2 is utilized during different types of underwater activity, would help increase our knowledge about the diving capacities and limitations of free-ranging animals. For example, studies combining this specific information with diving behavioural data recorded using tracking devices, would help improve bio-energetics models aimed to estimate daily or specific energetic requirements of individual animals or populations.

Consequently, the respiratory function and energetic requirements in marine mammals are central topics to be further explored. The information gained from future studies would not only be of interest for physiologists, but are also relevant for wildlife management and conservation. These studies could provide insights to better understand the behavioural decisions undertaken by marine mammals during natural diving or under disturbance exposure. Therefore, an increased research effort of these topics would improve predictions about how marine mammals respond to increasing anthropic impacts or potential changes in prey density or distribution. Such measurements would be of vital interest for those species that are facing increasing biotic and abiotic environmental changes, and that may impact their populations. Thus, this information will provide a

better understanding about whether a threatened species is operating at its maximum capacity, allowing predictions to be made about how these species may respond to environmental changes, as well as to evaluate their possible physiological plasticity to enhance survival. Ultimately, this information will assist to define improved management efforts and conservation priorities for the different marine mammal species.

1.5. List of symbols and abbreviations

- ADL = Aerobic dive limit
- cADL = Estimated or calculated aerobic dive limit
- DCS = Decompression sickness
- DMR = Diving metabolic rate
- FMR = Field metabolic rate
- f_R = Respiratory frequency
- TLC = Total lung capacity
- $\dot{V}$ = Respiratory flow
- VC = Vital capacity
- V_T = Tidal volume

1.6. Glossary

A

Aerobic dive limit
: Maximum dive duration that an air-breathing animal could reach by solely relying on the aerobic metabolic pathway and without showing an increase in blood lactate · 10

Alveolar collapse
: In diving mammals, it refers to the deflation of the alveoli as the air contained in the lungs is compressed with the increasing hydrostatic pressure at depth. · 4

B

Barotrauma
: Physical damage caused by a pressure difference between an internal gas compartment or the body, and the environmental gas or fluid. · 5

Bradycardia
: Decrease in the heart rate below the usual levels found while resting. · 9

C

Compliance
: In the respiratory physiology field, compliance is a synonym of flexibility. · 4

Compliant
: Relative to or characterized by compliance. · 4

D

Dead space
: In the respiratory field, the space in the respiratory system where gas exchange does not occur (e.g., trachea, bronchi, etc.). · 7

Decompression sickness
: Pressure-related disorder suffered by air-breathing divers frequently due to a fast change in environmental pressure that causes the formation of gas-bubbles in the blood and tissues. In humans, symptoms include fatigue, muscle and join pain, and if severe, paralysis, respiratory problems and death. · 5

G

Gas embolism
: Circulatory blockage caused by gas-bubble formation. · 5

Gas-bubble formation
: In air-breathing divers the accumulated inert gases in blood and tissues could come out from solution and form bubbles during or after a quick decrease in hydrostatic pressure (or decompression). · 5

H

Hypercapnia
: Condition produced by an increased level of CO_2 in the bloodstream which could lead to changes in the cardiorespiratory response. · 8

Hypoxia
: Condition produced by a reduced amount of O_2 in the blood, tissues and cells of an organism, which could lead to compromise their proper function. · 8

I

Inert gas narcosis
: Reversible alteration caused by the anesthetic effect of certain lipid-soluble gases at high pressures. In air-breathing divers narcosis is related with N_2 accumulation and could ultimately result in consciousness loss. · 5

L

Lung squeeze
: Pulmonary edema caused by intrathoracic pressures that are lower than environmental pressures during breath-hold diving. · 4

M

Minute ventilation
: The volume of air inhaled or exhaled per minute. It is estimated as the product of the tidal volume and the breathing frequency · 7

P

Peripheral vasoconstriction
: Cardiovascular response where the blood flow is restricted to certain tissues or organs. · 9

Pulmonary shunt
: Amount of blood bypassing the lungs and not participating in gas exchange. It ranges from 0% (inflated lungs fully participating in gas exchange) to 100% (collapsed lungs where gas exchange has ceased). · 5

R

Residual volume
: The amount of air remaining in the lungs following a maximal exhalation. · 7

Respiratory flow
: Speed of air coming in and out of the lungs during a respiratory maneuver. In human medicine it is usually reported in l min^{-1}. · 5

T

Tidal volume
: The amount of air moved in or out of the lungs with each respiratory cycle during normal respiration. · 7

Total lung capacity
: Total volume of air in the lungs after a maximal inspiration. · 7

V

Vital capacity
: The maximum volume of air that can be exhaled following a maximum inspiration. · 7

1.7. References

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2.This study

Humpback whale, *Megaptera novaeangliae* (Borowski, 1781)
Iceland, Atlantic Ocean, 2018
Photo credits: Joan Josep Soto Àngel

2. This study

The objective with the work presented in this thesis was to improve the current scientific knowledge about marine mammal respiratory and metabolic function. The thesis has been made possible with the collaboration between the **Marine Biology laboratory** (Zoology Department, Faculty of Biology) at **Universitat de València** (Valencia, Spain), and the **Comparative Physiology Laboratory** (Research Department) at **Fundación Oceanogràfic de la Comunitat Valenciana** (Valencia, Spain), which are, respectively, led by **Dr. Romana Capaccioni Azzati** and **Dr. Andreas Fahlman**, both co-supervisors of the research work presented here. The completion of each objective in the present thesis was possible through external grant funding, and the details for each funding award are provided in the Objectives subsection.

This study was aimed to investigate respiratory function and metabolic requirements of living animals under controlled experimental conditions. This allowed to collect data in animals that decided their participation in the experimental trials, which helped reduce stress and provided data for animals in a normal physiological state. The work performed here was made possible by collaboration with facilities that keep marine mammals under professional care. The Oceanogràfic (Valencia, Spain) and Siegfried and Roy's Secret Garden and Dolphin Habitat (Las Vegas, USA) provided access to animals and paid for all the costs associated with their care and training. The contribution of each facility is detailed in the Objectives subsection.

In addition, during the completion of the present thesis, I have had the opportunity to participate in a number of projects that provided research and field experience with both, wild marine mammals and those undergoing rehabilitation in human care. I was privileged to participate in the annual bottlenose dolphin population health assessment supervised by **Dr. Randall Wells** and carried out by the **Chicago Zoological Society's Sarasota Dolphin Research Program** in Sarasota Bay (Florida, USA), during two independent field sampling sessions in 2017 and 2018 that were funded by Fundación Oceanogràfic de la Comunitat Valenciana. Additionally, I took part on the PhD mobility program at Universitat de València, which encourages the transference of knowledge between research institutions and Universities worldwide, to help develop research capabilities during the early career of young scientists. Inside this program, I was privileged to collaborate with the **Zeehondencentrum Pieterburen** (Pieterburen, The

Netherlands) and the **Groningen University** (Groningen, The Netherlands) to accomplish 6 months of visit studentship for working with the harbour seal. This studentship was directly supervised by **DVM Ana Rubio García** and **Dr. Antonius G. G. Groothuis** and funded by Universitat de València (Vicerectorat d'Internacionalització i Cooperació and European Erasmus+ program), Zeehondencentrum Pieterburen, Fundación Oceanogràfic de la Comunitat Valenciana, and Clear Reef Social Fund. These two additional collaborations with external institutions provided the opportunity to assist on data collection of lung function in wild animals, which helped to better appreciate the limitations during field work and the problems surrounding conservation research and execution in these species.

2.1. Objectives

The central objective of the present thesis was to provide novel information about basic physiology through the study of respiratory function and energetic requirements in marine mammals. The acquired knowledge would increase the current understanding about respiratory physiology and energetics in these group of mammals, and would help understand their physiological limitations and how they may affect survival in a changing environment. This central objective was divided into specific investigations to explore research questions related with lacking knowledge in both fields. Two of these investigations focused to understand respiratory function and mechanics, and energy requirements of a threatened pinniped species. The third investigation used previously developed equipment for measuring lung function in cetaceans, to evaluate if this method could be used as a non-invasive diagnostic tool to assess respiratory health and monitor treatment efficacy. These investigations were accomplished through specific objectives that are presented in three independent chapters briefly summarized below.

Chapter I: Lung function in the Pacific walrus

This study aimed to **measure basic lung function and respiratory mechanics to define the respiratory capacity in adult individuals of Pacific walruses.** In addition, **respiratory parameters were measured in different body positions on land and in water to evaluate the possible effect of the pressure on the chest** while on land on measured variables. This investigation was performed at Oceanogràfic (Valencia, Spain), and specific funding for this project was provided by the Office of Naval Research to Dr. Andreas Fahlman (ONR YIP Award no. N000141410563).

Chapter II: Spirometry as a diagnostic tool in the bottlenose dolphin

The main objective of this investigation was to **evaluate the use of spirometry (or pulmonary function testing) as a non-invasive diagnostic tool in the bottlenose dolphin**. Lung function data were used to develop quantitative indices, based on those used in human medicine, but adapted for the specific respiratory function in cetaceans to evaluate lung health. In addition, these **indices provided preliminary explanations about the possible consequences of pulmonary disease on lung function**. This study was performed at Siegfried and Roy's Secret Garden and Dolphin Habitat which provided additional travel support to perform data collection, whereas the Office of Naval Research provided funding to Dr. Andreas Fahlman to perform the research study (ONR YIP Award No. N000141410563).

Chapter III: Metabolic rates in the Pacific walrus

This study aimed to **evaluate and determine the metabolic rates for specific in-water activities in adult Pacific walruses.** Specifically, the investigation aimed to measure the **energetic cost during short and shallow inactive diving and subsurface swimming, and** to compare these results with measurements **while floating at the water surface**. This objective was performed at Oceanogràfic and specific funding was provided by the United States Geological Survey Changing Arctic Ecosystems Initiative to Dr. Andreas Fahlman (Award# G18AC00011).

2.2. Thesis structure

The present thesis is structured following the regulation for doctoral dissertations at Universitat de València (Reial decret 99/2011 Modificat pel Consell de Govern 28-VI-2016). The thesis includes the previously outlined introductory section, which has aimed to justify the present investigation by providing a general chronological overview of the main findings that highlighted the relevance of studying respiratory physiology and energetic requirements in marine mammals to help with their conservation. The following section provides a general description about the methodology used to successfully measure physiological parameters when working with animals under controlled conditions, and describes the equipment, calibration, and data processing. This section is then followed by the core of the present thesis, which is composed of three chapters describing the completion of the specific objectives intended in this investigation. These chapters are organized following the regular subsections of scientific manuscripts,

including a brief introduction describing the specific background related with each objective, a detailed methodology, the description of the main results, and the discussion of the main findings with a summary of conclusions and the references list. These three chapters have been adapted for their publication in international peer reviewed scientific journals. For each of these chapters, the list of authors, the corresponding institutions involved during the publication process, and the details for accessing the published work are provided in each subsequent chapter. Following these three chapters, there is a final section that summarizes the main conclusions obtained from the present thesis, and future suggestions derived from the present investigation. In addition, the present thesis includes an extended summary of the investigation translated into “castellano”, one of the official languages at the Universitat de València.

3. General methodology

Sperm whale, *Physeter macrocephalus*, Linnaeus, 1758
Azores, Atlantic Ocean, 2014
Photo credits: Andreas Fahlman

3. General methodology

3.1. Requirements for animal research

The studies described in this thesis included experiments on live animals permanently housed under professional care at the different facilities specified in the Objectives subsection. The participating facilities have been inspected to comply with both national and international standards for animal welfare and management. Each species was maintained under a determined range of environmental conditions while controlling comfort and quality of animal life. The food provided was certified human food grade and regularly sampled for nutritional quality and energetic content.

The animals were desensitized to the research equipment and trained to perform the experimental procedures. During both training sessions or research trials, the animals could decide whether they wanted to participate or to end a session or trial at any point. The animals were not confined or restrained during any of the experimental procedures. This methodology allowed for data collection under a stable and relaxed physiological state while ensuring animal welfare. All experimental procedures were evaluated and approved by the Animal Welfare Committee at Oceanogràfic (Valencia), which was composed by internal and external experts accredited to examine proposals involving the use of animals in research following the European legislation. The revised experimental procedures were then submitted for accurate revision and final approval by the local government (Conselleria de Agricultura, Desenvolupament Rural, Emergència Climàtica i Transició Ecològica). Additional animal care approvals were obtained to comply with external funding institutions and are detailed in the Materials and methods sections of Chapters I-III.

3.2. Animal management and training

The species studied in the present thesis included the Pacific walrus [*Odobenus rosmarus divergens* (Illiger, 1815)] and the bottlenose dolphin [*Tursiops truncatus*, (Montagu, 1821)]. The Pacific walruses were housed at Oceanogràfic (Valencia, Spain), while the bottlenose dolphins were housed at Siegfried and Roy's Secret Garden and Dolphin Habitat (Las Vegas, USA). Both facilities have several sea-water pools, and the walruses were also provided with access to an area to haul out where they could rest or undergo medical or training procedures. The animals were provided with a variable diet

mainly composed of a mixture of fish (capelin, mackerel, herring, etc.) for dolphins, and a mixture of fish and molluscs (squid, mussels, etc.) for the walruses. The animals were supplemented with vitamins and additional gelatine ingestion.

Complete desensitization and training for the experimental procedures were achieved after continuous and extensive periods prior to initiating data collection. The required behaviours for each research objective were accomplished by small approximations using operant conditioning and positive reinforcement techniques (Laule, 2010). Once the desirable behaviour was achieved, the intervals between reinforcements were progressively increased during periods where the animals were calm, relaxed, and focused on the experimental procedures. This allowed to set the appropriate extended periods required for physiological data collection. In general, the criteria for accepting a specific experimental behaviour as achieved included: calm animals breathing normally and avoiding interaction with the equipment or the surrounding environment. The specific behaviours required for each objective in the present thesis are detailed in the corresponding Materials and methods sections in Chapters I-III. For those experiments that required minimal food intake during data collection, the food reinforcement was progressively lowered and replaced by other stimuli. All training sessions and experimental trials during data collection were performed in a relaxed environment at each facility to ensure reliable results. Communication between animal care specialists and researchers continued throughout this investigation to allow a clear understanding about the specific requirements for each experimental procedure and to assure animal welfare.

3.3. Equipment, measurements, and data processing

In this section, a general summary of the experimental components, calibration procedures for data collection, and data processing are provided. Additional and detailed information of the experimental set up, experimental design, studied individuals, and data processing and curation are provided for each specific experimental objective in the Materials and methods sections of Chapters I-III.

3.3.1. Lung function studies in walruses and dolphins (Chapters I and II)

Equipment: general components, maintenance, and data collection

For the completion of the objectives in Chapters I and II in the present thesis, previously developed and validated equipment to measure lung function in pinnipeds and

cetaceans was used (Fahlman et al., 2015; Fahlman et al., 2019; Fahlman et al., 2020; Fahlman and Madigan, 2016). Lung function studies were performed by measuring respiratory flow rate ($\dot{V}$, l s^{-1}) using custom-made Fleisch type pneumotachometers specifically adapted for the studied species. Fleisch type pneumotachometers are commonly cylindrical structures housing a matrix built by assembling parallel small capillary tubes. This matrix creates a resistance to the air passing through, while allowing for a laminar or pseudo-laminar airflow through the system (Zock, 1981). The characteristics of this type of pneumotachometers allow for a proportional, and nearly linear relationship between the airflow passing through the system and the created resistance (Zock, 1981). The principle of this relationship relies on Poiseuille's law, which states that the flow rate of a fluid is proportional to the pressure lost per unit of length of the resistive element (Equation 1).

$$\text{Equation 1: } \frac{\Delta P}{\dot{V}} = \frac{8 \cdot \eta \cdot L}{\pi \cdot r^4}$$

Where ΔP is the variation in the pressure between both sides of the matrix, η is the viscosity coefficient of the gas, and L and r are, respectively, the length of the resistive element and the radius of the cylindrical system. Therefore, the magnitude of the resistance depends on the $\dot{V}$ or velocity of air passing through the matrix, the air viscosity, and the characteristics of the pneumotachometer. Since the dimension of a given pneumotachometer is constant, and the viscosity and composition of respiratory gases varies slightly at pressures encountered during physiological measurements (Turner et al., 1989), the resistance of the matrix would be proportional to the $\dot{V}$ at constant temperatures (Tang et al., 2003). This resistance could be determined by measuring the differential pressure created on both sides of the matrix while the air is passing through the system.

In the present thesis, the pneumotachometers (or spirometers) were designed to comfortably fit over the nostrils of the walruses and the blowhole of the dolphins (Figure 3.1). The end of the pneumotachometer contained a silicone ring at the base to allow for a soft placement over the respiratory airway openings while ensuring a proper seal of the system (Figure 3.1). The custom-made pneumotachometers housed a low-resistance laminar flow matrix of parallel capillary tubes (Item #Z9A887-2, Merriam Process Technologies, Cleveland, OH, USA, Figure 3.1.).

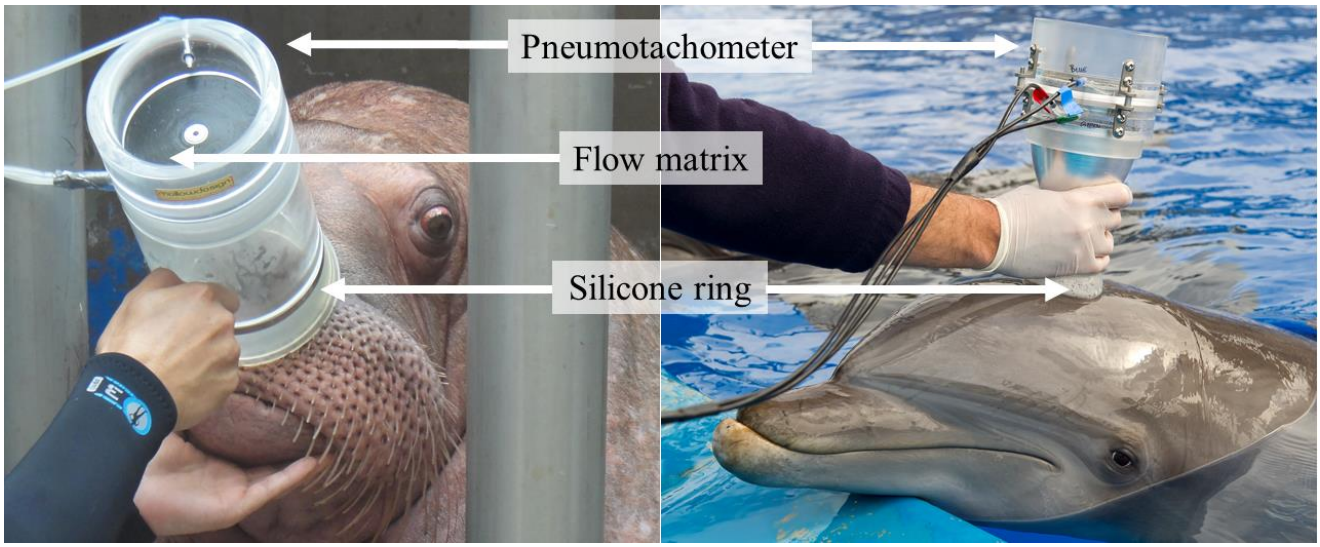

Figure 3.1. Pneumotachometer for lung function testing.
Examples of custom-made pneumotachometers or spirometers adapted for measuring respiratory flow in Pacific walruses (left) and bottlenose dolphins (right). Pictures provided by Oceanogràfic, Ciudad de las Artes y las Ciencias (Valencia, Spain).

When the animals breathed into the pneumotachometer, the resistance created by the $\dot{V}$ was measured with a differential pressure transducer (Spirometer Pod, ML 311, ADInstruments, Colorado Springs, CO, USA). This differential pressure transducer was connected to the pneumotachometer via two ports placed on each side of the flow matrix (Figure 3.2), which were positioned at each side of the matrix to generate a negative deflection for the expiratory phase. The differential pressure transducer converted measured differential pressures into an electrical signal (voltage) by assuming a proportional and linear relationship. These data were captured as a function of time at 400 Hz using a data acquisition system (Powerlab 8/35, ADInstruments), and displayed in a laptop computer while running physiological data analysis software (LabChart, v. 8.1, ADInstruments, Figure 3.2). This software provided measured raw $\dot{V}$ in volts as a function of time (V s^{-1}). Both, the pneumotachometer and the laminar flow matrix were cleaned and disinfected between each experimental trial by submerging these components in a Virkon™ S solution (~1% by mass) for 10 minutes. The pneumotachometer and the laminar flow matrix were dried before each experimental trial and visually examined to prevent the possible obstruction of the matrix.

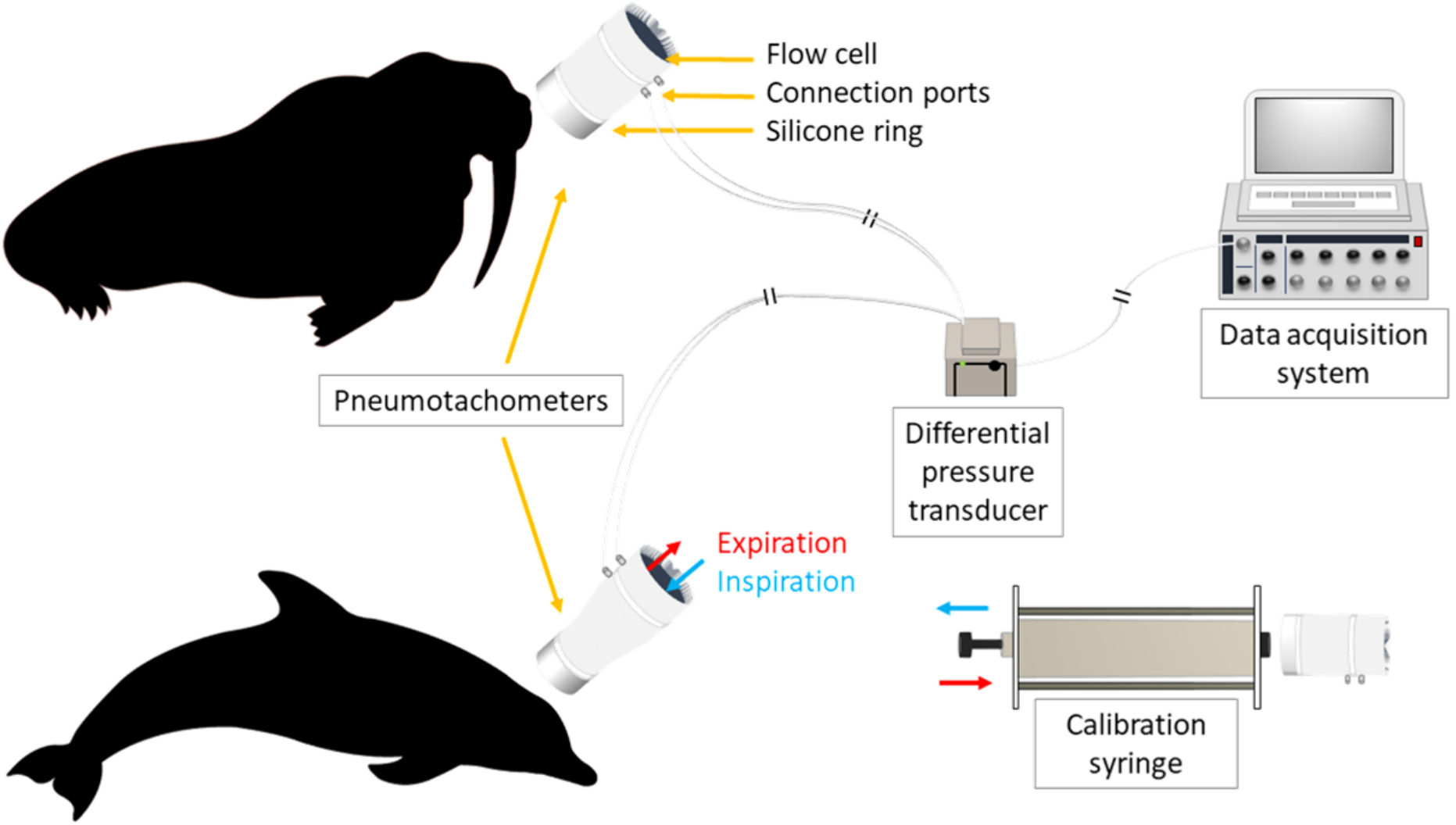

Figure 3.9. Lung function testing components.
Schematic diagram (unscaled) of the lung function sampling and calibration components used while measuring respiratory flow in Pacific walruses and bottlenose dolphins. The grey solid lines represent the sampling connections between the different components described in this section. The arrows in the dolphin's pneumotachograph show the air direction for the expiratory (red) and inspiratory (blue) phases. The position of the pneumotachometer and the calibration syringe for the calibration procedures (see following section) is also represented. The arrows placed at the pumping handle of the calibration syringe show the direction of the simulated respiratory phases (expiration: red; inspiration: blue).

Calibration

The relationship between measured differential pressures and $\dot{V}$ was determined using a 7 l calibration syringe (Series 4900, Hans-Rudolph Inc., Shawnee, KS, USA) and assuming a proportional and linear response between airflow, measured differential pressure, and voltage. For the calibration procedure, the pneumotachometer was placed at the opening of the syringe and various pumping cycles of air were performed in both directions (Figure 3.2; Tang et al., 2003). From these recorded calibration manoeuvres, calibration factors were determined using the measured raw airflow and the known volume of the calibration syringe. The design of the housing structure of the pneumotachometers (non-symmetrical) and the opposing dynamics while pushing and pulling air into the syringe, required the use of separate calibration factors for the expiratory and inspiratory phases (Fahlman et al., 2015). In addition, since the animals performed respiratory manoeuvres of different respiratory effort, diverse pump cycles of various flow rates were performed during the calibration to determine calibration factors for the diverse types of measured respiratory manoeuvres. The measured raw flow as a function of time during calibration manoeuvres was integrated to yield the voltage that corresponded to the 7 l moved in or out the pneumotachometer by the calibration syringe. The calibration factors were then computed by dividing the pumped volume by the reached voltage during the calibration manoeuvres (see an example in Figure 3.3). The estimated $\dot{V}$ was then calculated by multiplying the obtained calibration factors by measured raw $\dot{V}$ during data collection measurements.

The calibration of the pneumotachometer was repeated before and after each experimental trial, which also helped confirm the linear response of the pneumotachometer (Figure 3.4). The flow baseline was zeroed before each calibration cycle and experimental trial. Baseline drift was prevented by injecting dry pressurized air into the sampling connections to avoid water condensation in the tube measuring the pressure. While linear relationships between $\dot{V}$ and differential pressures were assumed for all flow rates, the higher $\dot{V}$ reached by the animals during some respiratory manoeuvres produced a non-linear relationship which could lead to overestimations of $\dot{V}$ (Fahlman et al., 2015). In addition, although the pump cycles during the calibration aimed to reproduce the respiratory manoeuvres performed by the animals, the inability to replicate some high $\dot{V}$ could have resulted in an overestimation of the obtained $\dot{V}$ for the larger respiratory manoeuvres.

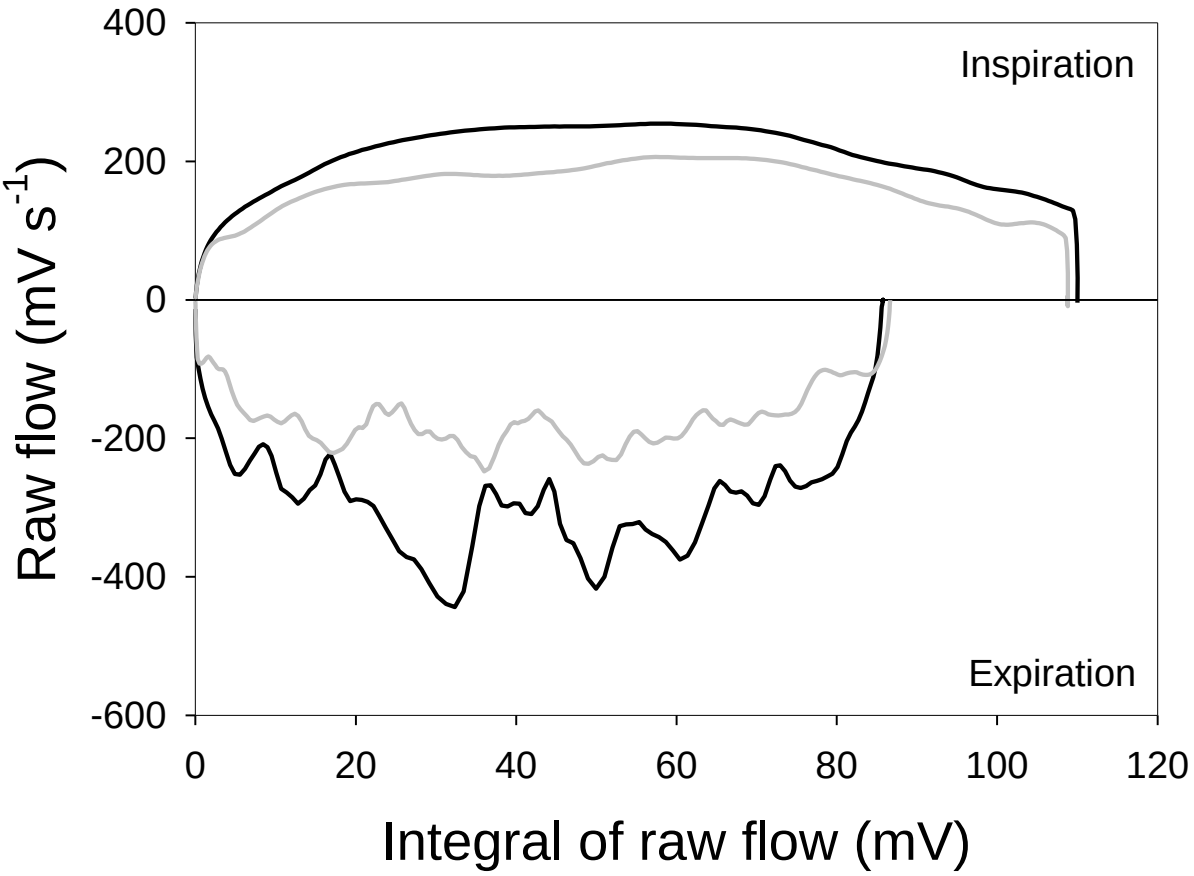

Figure 3.13. Pneumotachometer: calibration.

Examples of performed calibration pump cycles simulating spontaneous (grey lines) and maximal (black lines) respiratory manoeuvres while using a pneumotachometer designed for walruses and a 7 l calibration syringe. Measured raw flows were integrated to obtain the voltage that corresponded to the pumped volume of the calibration syringe. The calibration factors were obtained by dividing the known volume of the syringe by the obtained voltage value for each manoeuvre. As an example, the voltage corresponding with the maximal calibration manoeuvre during the expiratory phase was 85.78 mV, and the computed calibration factor 81.60 l V^{-1}.

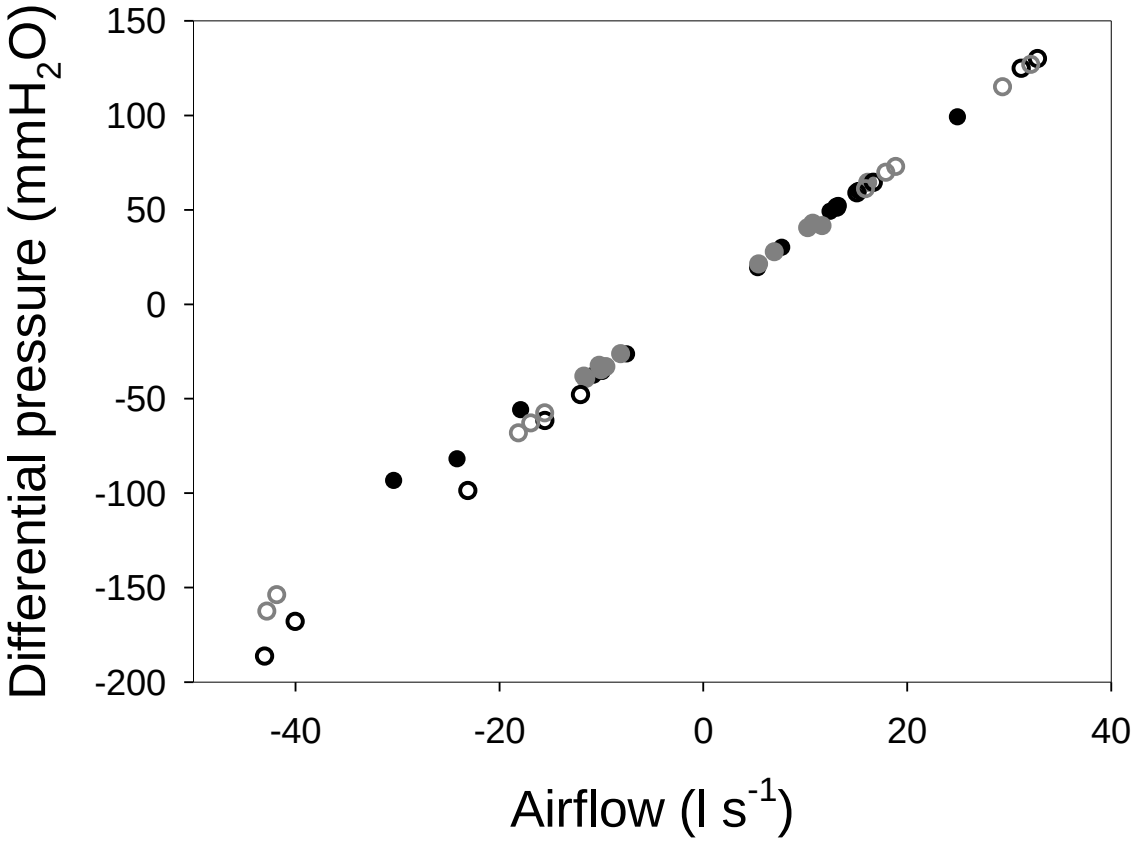

Figure 3.21. Pneumotachometer: linear response.
Representation of measured differential pressure and obtained calibrated airflow after performing several pump cycles using a 7 l calibration syringe and a pneumotachometer designed for walruses (filled circles) and for dolphins (open circles). Each point represents the maximum values obtained for each calibration manoeuvre. The calibration manoeuvres were performed before (black) and after (grey) each lung function measurement. The calibrated airflow was calculated multiplying the measured raw airflow by the calibration factor obtained for each calibration manoeuvre. Negative and positive values represent, respectively, the expiratory and inspiratory phases.

Data processing

For each experimental trial, obtained calibration factors were independently used for each respiratory phase and manoeuvre based on the similarity between calibration and respiratory manoeuvres. For homogeneous sections of comparable respiratory effort, an average of computed calibration factors obtained from similar calibration manoeuvres was often used. The raw $\dot{V}$ was separated into the expiratory and inspiratory signals, and the selected calibration factors for each respiratory phase were multiplied by the raw signal to isolated manoeuvres or homogeneous sections. The obtained calibrated expiratory ($\dot{V}_{exp}$) and inspiratory ($\dot{V}_{insp}$) $\dot{V}$ were then pooled together to generate calibrated

$\dot{V}$. This combined and calibrated $\dot{V}$ was cleaned using a low-pass filter (cut-off frequency 10Hz) and a transition width (sharpness, 50Hz). These filters reject high-frequency components derived from turbulent signals and attenuate brief peaks in the flow signal. Then, the respiratory tidal volume (V_T, l) was determined through the integration of the calibrated $\dot{V}$ curve over time (Figure 3.5; Grenvik et al., 1966).

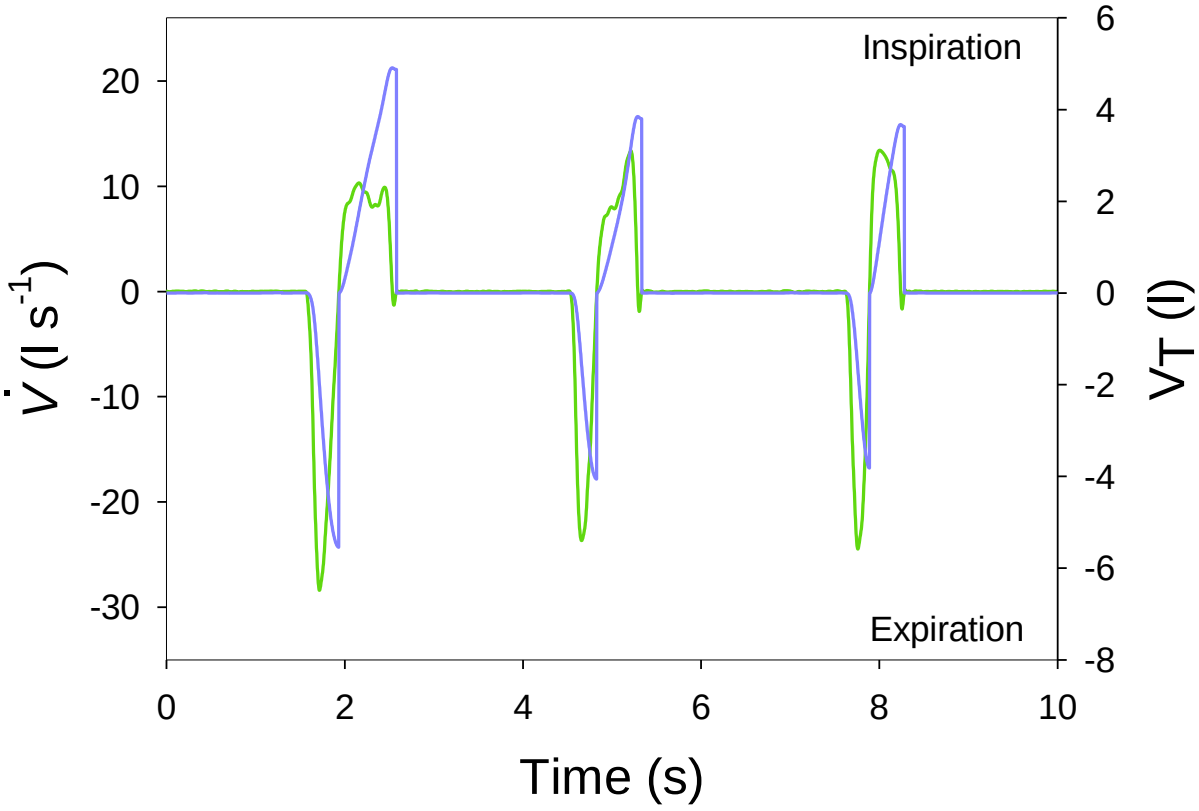

Figure 3.25. Computed tidal volume from calibrated respiratory flow in lung function studies.
Example representation of measured spontaneous respiratory manoeuvres over time from dolphins. In marine mammals, the breathing pattern starts with an expiration (negative values) followed by an inspiration (positive values). The tidal volume (V_T, blue line) was computed by integrating the curve of calibrated respiratory flow ($\dot{V}$, green line).

Then, estimated V_T was converted to account for the differences in ambient pressure, temperature, and humidity during measurements. Inhaled V_T during lung function studies is measured in ambient temperature and pressure conditions (ATP), which are different to those within the lungs. The air contained in the lungs in mammals is considered to be at 37^{o}C and 100% saturated with water vapour (body temperature and pressure saturated conditions, BTPS). Since the volume of a determined gas varies with temperature and pressure, a correction factor should be applied to measured expiratory

(V_{Texp}) and inspiratory (V_{Tinsp}) V_T to account for this volume change and avoid variation in the volume (Quanjer et al., 1993). In human medicine, lung function measurements are performed under standardized environmental conditions where measured V_T is normally reported in BTPS (García-Río et al., 2013; Miller et al., 2005). However, studies comparing animals of different taxa should consider the diverse body temperature ranges and the variety of environmental conditions where respiratory data could be collected. Thus, the results in the present study are reported in standard temperature (273.15 K) and pressure (101.325 kPa) dry (0% relative humidity) conditions (STPD) to allow for comparison between different species and locations. Therefore, V_{Texp} was corrected from BTPS conditions (Equation 2), while V_{Tinsp} was corrected from ATP conditions and for the presence of ambient water vapor pressure (Equation 3):

$$\text{Equation 2: } V_{Texp(STPD)} = V_{Texp(BTPS)} \cdot T_{Standard} \cdot \frac{P_{Ambient} - P_{H_2O(Animal)}}{T_{Animal} \times P_{Standard}}$$

$$\text{Equation 3: } V_{Tinsp(STPD)} = V_{Tinsp(ATP)} \cdot T_{Standard} \cdot \frac{P_{Ambient} - P_{H_2O(Ambient)}}{T_{Ambient} \times P_{Standard}}$$

Where $P_{Ambient}$ (ambient pressure in kPa), $T_{Ambient}$ (ambient temperature in K), and humidity were independently measured for each experimental trial. For Equation 2, T_{Animal} was assumed to be 37ºC (Miller et al., 2005) with a water vapor saturation inside the lungs of 100% ($P_{H2O(Animal)}$ = 6.25 kPa, Miller et al., 2005). For Equation 3, $P_{H2O(Ambient)}$ (ambient water vapor pressure in kPa) was computed using measured ambient humidity (AH) and temperature (T), and the estimated dew point. Dew point was calculated using Equation 4 (Equation 8 in Lawrence, 2005).

$$\text{Equation 4: Dew point} = \frac{B \cdot \left[\ln\left(\frac{AH}{100}\right) + \left(\frac{A \cdot T}{B+T}\right)\right]}{17.625 - \left[\ln\left(\frac{AH}{100}\right) - \left(\frac{A \cdot T}{B+T}\right)\right]}$$

Where A and B coefficients were 17.625 and 243.04, respectively, as recommended for $P_{H2O(Ambient)}$ estimations of environmental temperatures from -40ºC to 50ºC with a relative error of < 0.4% (Alduchov and Eskridge, 1996). Then, $P_{H2O(Ambient)}$ in the inhaled air was computed using Equation 5 (Equation 21 in Alduchov and Eskridge, 1996).

$$\text{Equation 5: } P_{H_2O(Ambient)} = 6.1094 \cdot e^{\left(\frac{17.625 \cdot \text{dew point}}{243.04 + \text{dew point}}\right)}$$

Once volume calibration factors were computed, the estimated V_{Texp} and V_{Tinsp} were separated and multiplied, respectively, for the obtained calibration factors from Equations 2 and 3. Obtained V_{Texp} and V_{Tinsp} were then combined to yield corrected V_T. Then, lung function parameters were obtained breath by breath using LabChart. The $\dot{V}_{exp}$ $\dot{V}_{insp}$, V_{Texp}, and V_{Tinsp} were calculated as the maximum recorded values measured for each respiratory phase from the calibrated and filtered $\dot{V}$ and corrected V_T. The expiratory duration (T_{exp}) was defined as the time elapsed from the flow decreased below to zero at the beginning of the breath, until the flow returned to zero, where the inspiration began (Figure 3.5). The inspiratory duration (T_{insp}) was defined as the time elapsed from end of expiration until the flow returned to zero at the end of the respiratory manoeuvre (Figure 3.5). The total duration (T_{tot}) of each breath was calculated by summing the duration of each respiratory phase.

3.3.2. Metabolic studies in walruses (Chapter III)

Equipment: general components, maintenance, and data collection

The objectives in Chapter III were accomplished using the most common configuration for metabolic measurements in large animals: flow-through respirometry (Lighton, 2008). This methodology yields information about the amount of O_2 consumed by the animal inside a respirometer, which is used an index of metabolic rate. In Chapter III, the experimental animals were maintained inside a respirometer while measuring the O_2 consumption ($\dot{V}O_2$, l O_2 min^{-1}) and CO_2 production ($\dot{V}CO_2$, l CO_2 min^{-1}) rates to estimate the metabolic rate. The respirometer used was a Plexiglas dome adapted for the walruses, and metabolic measurements were performed with the animals floating at the water surface of a sea-water pool (Figure 3.6). The respirometer was connected to a vacuum pump (FlowKit Mass Flow Generator, FK-500-l, Sable Systems Int, Las Vegas, NV, USA) via a corrugated tube that pulled ambient air through the dome at a fixed flow rate of 500 l min^{-1} (Figures 3.6 and 3.7).

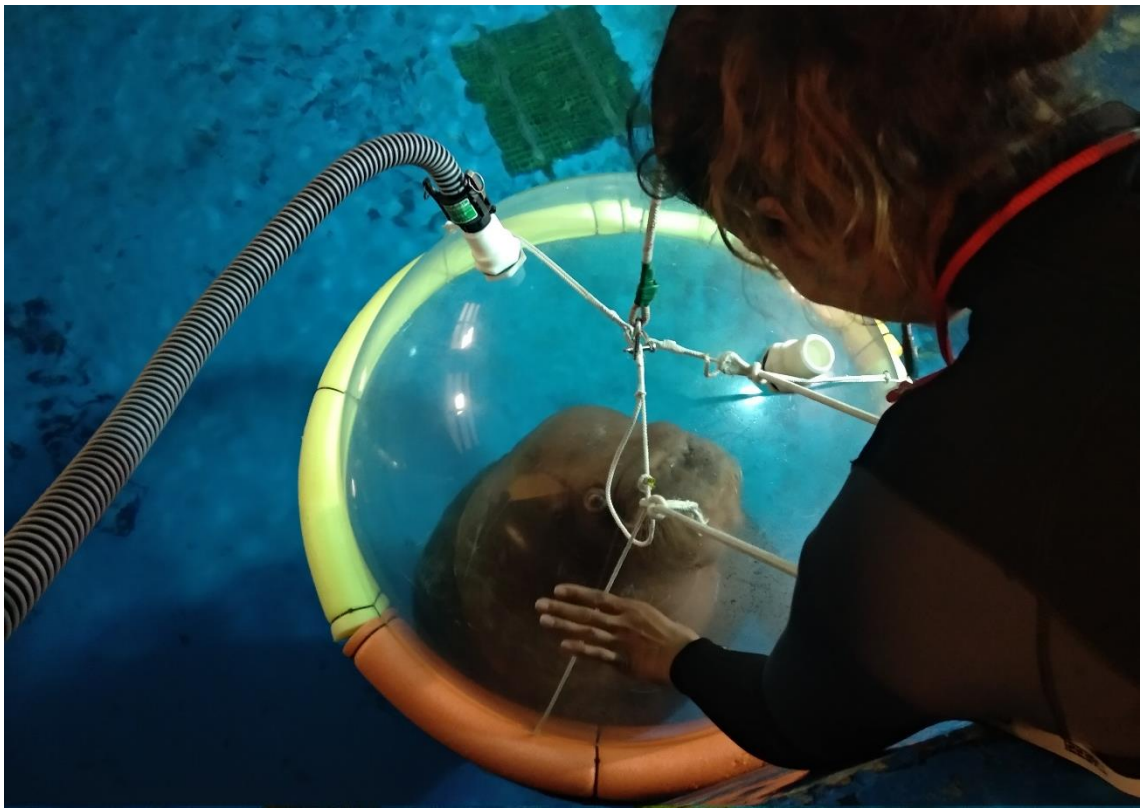

Figure 3.34. Metabolic session using flow-through respirometry in walruses. The respirometry dome was made buoyant by attaching polyethylene foam to the base, and was placed at the water surface with the walrus surfacing inside to measure metabolic rates. The dome was connected via a corrugated tube to a vacum puump that pulled ambient air into the system through an inlet apperture. Picture provided by Oceanogràfic, Ciudad de las Artes y las Ciencias (Valencia, Spain).

The O_2 and CO_2 content of the excurrent air were measured with a fast-response gas analyser (Gemini Respiratory Gas Analyzer, part no. 14-1000, CWE Inc., Allentown, PA, USA) that took a subsample of the excurrent gas. This subsample was passed through a hydrophobic filter that prevents condensed water from entering the gas analyser, followed by a Nafion© line connected to the gas analyser (Figure 3.7). Nafion© is a highly selective, semi-permeable membrane to water vapor that equalizes the sample stream humidity with the ambient humidity. The air flow rates and gas concentrations were captured as a function of time at 400 Hz using a data acquisition system (Powerlab 8/35, ADInstruments), and displayed in a laptop computer while running physiological data analysis software (LabChart, ADInstruments, Figure 3.7). This software provided measured raw airflow in l min^{-1} and the gas content as percentage. The equipment was allowed to warm up from 5 to 10 minutes prior to data collection per manufacturer specifications of the fast-response gas analyser. The respirometry dome was cleaned and disinfected with alcohol between each experimental trial.

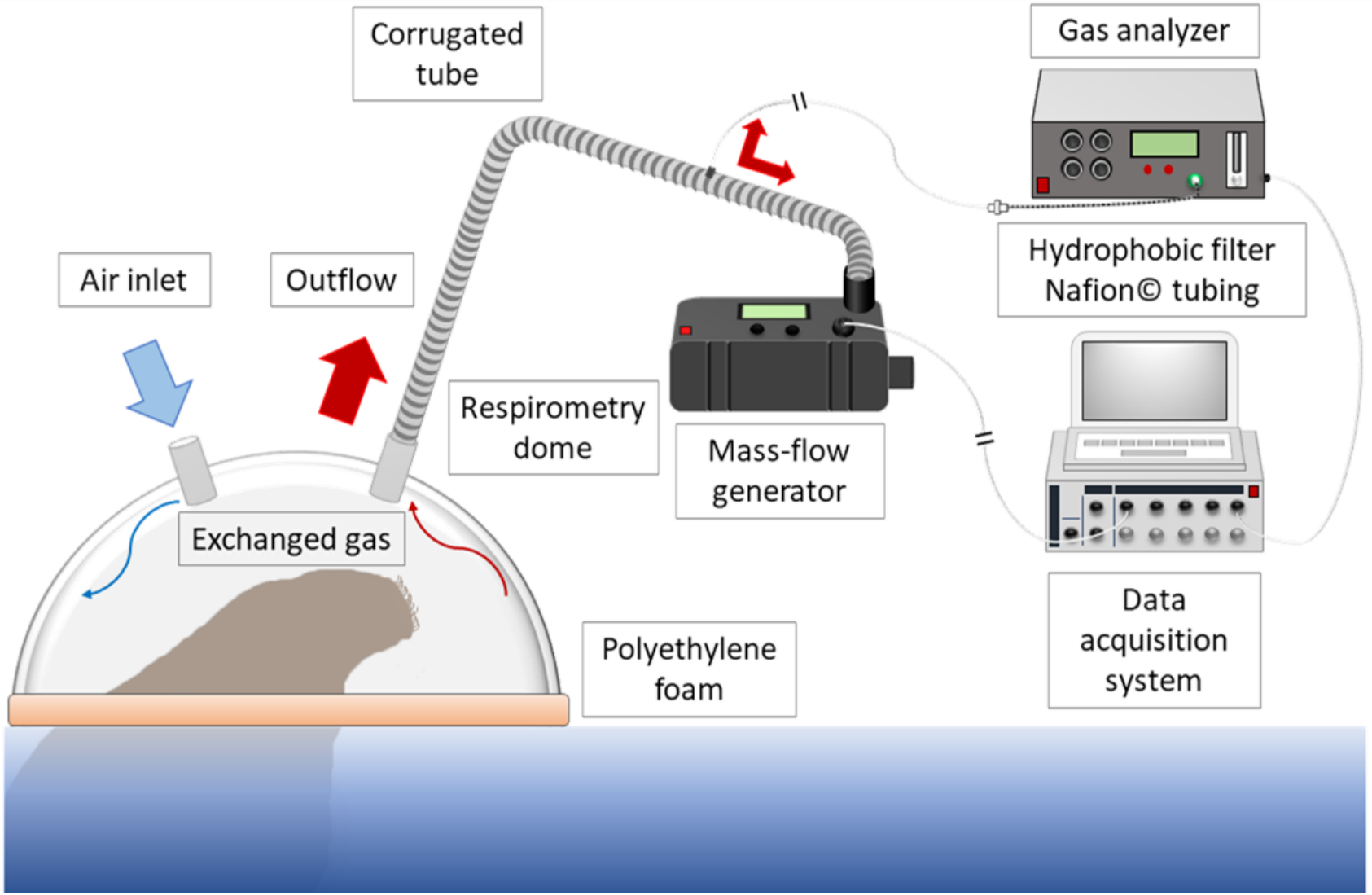

Figure 3.43. Open flow-through respirometry components.
Schematic diagram (unscaled) of the equipment set up used in the present study while measuring O_2 consumption and CO_2 production rates. The arrows show the direction of the air through the system. The grey solid lines represent the sampling connections between the different components described in this section.

Calibration

The gas analyser was routinely calibrated using ambient air and a commercial gas mixture (5% O_2, 5% CO_2, and 90% N_2; UN1956 Air Liquide, USA). A simultaneous CO_2 and O_2 dilution test was conducted to evaluate the system for possible leaks, and to assess the accuracy in measured O_2 and CO_2 concentrations. This method is based on the N_2 dilution technique (Fedak et al., 1981), and consists in flowing a determined gas at a known rate and concentration into the respirometer when the animal is absent. The volume of the gases injected would be added to the environmental air pulled through the system. Therefore, the measured increase in environmental O_2 and CO_2 concentrations can be compared to that predicted by the flow rate of gas into the respirometer. A commercial gas mixture (10% CO_2 and 5% O_2) was flowed into the system at two different flow rates of 10 and 15 l min^{-1}. Both flow rates were tested during two independent tests while the pump pulled air through the system at 190 and 100 l min^{-1} to evaluate the accuracy for both flow rates. The predicted concentrations of CO_2 and O_2 flowed into the system were estimated using Equations 6 and 7, respectively.

Equation 6:

$$\text{Predicted } [CO_2] = \frac{(\text{Cal flow} \cdot F_{i\,CO_2\,cal}) + (\text{System flow} \cdot F_{i\,CO_2\,env})}{(\text{Cal flow} + \text{System flow}) \cdot 100}$$

Equation 7:

$$\text{Predicted } [O_2] = \frac{(\text{Cal flow} \cdot F_{i\,O_2\,cal}) + (\text{System flow} \cdot F_{i\,O_2\,env})}{(\text{Cal flow} + \text{System flow}) \cdot 100}$$

Where Cal flow and System flow refer to the flow rates of the commercial gas mixture being flowed into the system, and the flow of excurrent air pulled by the pump, respectively, while F_i is the incurrent fraction of calibration (cal) or environmental (env) gases. The environmental gas concentrations were considered 0% and 21% for CO_2 and O_2 respectively. By applying Equation 6 and 7 for the different combination of described calibration and system flow rates, the measured O_2 and CO_2 concentrations were within 6% of estimated values. This leak test is additionally important to determine the lag time characteristics of the respirometry system (see following subsection).

Estimation of the respirometry system time response

The changes of the gas composition that the organism creates in the respirometry chamber during flow-through measurements are not instantly reflected in the excurrent air stream. The time response of the respirometry system relies on the effective or physical volume of all the components (respirometry dome, sampling tubes, etc.), the flow characteristics of each component, the response kinetics of the gas analysers, etc. The effective volume (EV, l) of a given configured respirometry system includes the volume and airflow rates of every component. Thus, the calculation of the EV could be used to estimate the response interval for detecting a change in the gas composition from the chamber where the organism is located. The calculation of the effective volume relies on the fact that for a given respirometry configuration, the approach of the fractional concentration of the gas in the excurrent air (F_e) to the new steady state is an exponential function of time assuming uniformly mixed gas in the respirometer (Bartholomew et al., 1981). Therefore, the rate in which F_e approaches the equilibrium is constant, and at a given time after a change in the gas composition, the F_e will have changed by a constant fraction (Z) of the distance to the new equilibrium value (Equation 8 and 9; Equations 1 and 2 in Bartholomew et al., 1981).

$$\text{Equation 8: } Z = 1\text{-}e^{\text{-Flow rate} \cdot \frac{\Delta T}{EV}}$$

$$\text{Equation 9: } Z = \frac{F_eT - F_eT_{-1}}{F_{eq} - F_eT_{-1}}$$

Where in Equation 8 ΔT is the time interval between successive samples, while in Equation 9 F_eT is the fractional excurrent concentration of the gas being measured at time T, F_eT_{-1} is the same concentration measured at time T_{-1}, and F_{eq} is the fractional final equilibrium of the excurrent gas concentration. Combining and rearranging Equations 8 and 9, the EV can be used to estimate the characteristics of the configured respirometry system. In the present study, the estimated EV of the respirometry system was 465 l, which was computed as the average of 10 different intervals of measured fractional excurrent O_2 and CO_2 concentrations for one leak test, and the same number of intervals for 3 experimental trials once the animal abandoned the respirometry dome.

Once the EV was computed, the time constant, or the time required by the system for responding to a 63% of a determined change (Bartholomew et al., 1981), could be calculated by dividing the EV by the flow rate pulled through the respirometry chamber

(Bartholomew et al., 1981). As the approach to the new steady state is an exponential function of time (see Equation 8), it is possible to estimate the time required by the system to reach a determined percentage of response from the new steady state using Equation 10 (Equation 8.1 in Lighton, 2008).

$$\text{Equation 10: } \text{Time} = - \frac{\text{EV}}{\text{Flow Rate}} \cdot \ln\left(\frac{100 - \%\ \text{of response}}{100}\right)$$

For most flow-through respirometry systems, the time response is reported as the necessary time to reach a 95% fractional transformation, which computing the logarithmic part of Equation 10 yields to a factor of ~3 to be multiplied to the time constant (Bartholomew et al., 1981; Fahlman et al., 2007). The estimation of the time constant therefore provides information about the time that the respirometry system requires to renew the air contained in the respirometry chamber. In the present study, the time constant was 0.93 min (465 l/500 l min^{-1}) and the time required to reach a 95% fraction transformation to a new steady state was 2.79 min (167 s, Figure 3.8).

Data processing

The calculation of the total volumes of consumed O_2 and produced CO_2 during a determined period of time was estimated using LabChart software (v. 8.1, ADInstruments). The vacuum pump or mass flow generator automatically corrected airflow rates to standard temperature and pressure conditions (STP). Measured flow rate (l min^{-1}) was then corrected for measured humidity inside the respirometry dome and converted to STPD using Equation 11 (Equation 8.6 in Lighton, 2008):

$$\text{Equation 11: } \text{Flow rate}_{STPD} = \text{Flow rate}_{STP} \cdot \left(\frac{P_{Ambient} - P_{H_2O(Ambient)}}{P_{Ambient}}\right)$$

Where $P_{Ambient}$ was measured for each experimental trial, and $P_{H2O(Ambient)}$ was calculated using Equation 4 and 5 as described early in this section for the lung function studies developed in Chapters I and II. Measured O_2 and CO_2 concentrations were baselined using measured ambient values at the end of the trial, and the differences in the analysers lag-time were adjusted to provide simultaneous changes from both measured gases. Corrected flow rates and measured gas concentrations were then multiplied to calculate the instantaneous $\dot{V}O_2$ and $\dot{V}CO_2$ (l s^{-1}). To compensate for the O_2 dilution effect produced by the CO_2 generated by the organism, the instantaneous $\dot{V}O_2$ and $\dot{V}CO_2$ were calculated following corrections suggested by Withers (1977), and adapted for pull flow-

through respirometry. These calculations are showed in Equation 12 and 13 (Equations 11.7 and 11.8 in Lighton, 2008):

Equation 12:

$$\dot{V}O_2 = \text{Flow rate}_{STPD} \cdot \frac{[(F_iO_2 - F_eO_2) - F_iO_2 \cdot (F_eCO_2 - F_iCO_2)]}{1 - F_iO_2}$$

Equation 13:

$$\dot{V}CO_2 = \text{Flow rate}_{STPD} \cdot \frac{[(F_eCO_2 - F_iCO_2) + F_iCO_2 \cdot (F_iO_2 - F_eO_2)]}{1 + F_iCO_2}$$

Where, for each measured gas (O_2 and CO_2) F_i denotes the fractional incurrent concentration and F_e the fractional excurrent concentration. The fractional incurrent concentrations for both, O_2 and CO_2 were included as measured ambient values for each experimental trial. The obtained instantaneous $\dot{V}O_2$ and $\dot{V}CO_2$ following corrections from Equations 12 and 13, respectively, where then integrated over determined periods to yield the total volume of the exchanged gases (see an example in Figure 3.8).

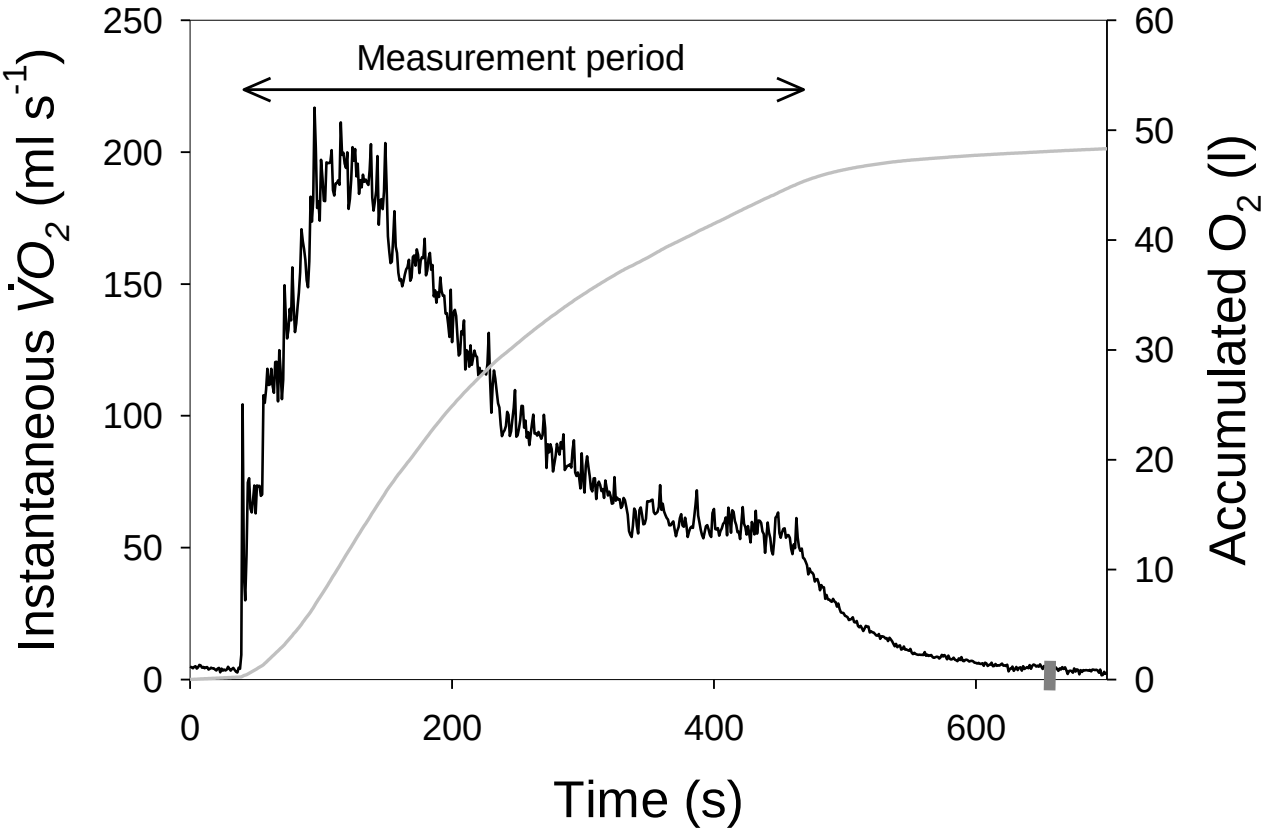

Figure 3.44. Calculation of total volume of consumed oxygen during one metabolic measurement in walruses.

Example of corrected instantaneous O_2 consumption rate ($\dot{V}O_2$, black line) during a metabolic measurement where one walrus remained inside the respirometry dome 7.2 min (from time = 38 s to time = 468 s, indicated as measurement period). Corrected instantaneous $\dot{V}O_2$ was obtained by applying Equation 12, using measured airflow converted to STPD conditions, and measured O_2 and CO_2 concentrations. Corrected instantaneous $\dot{V}O_2$ was then integrated to yield the total volume of consumed O_2 (grey line). The total volume of consumed O_2 in this example corresponds to the integral from the point where the walrus entered the dome, to the point where the instantaneous $\dot{V}O_2$ recovered ambient values (~650 s, marked with a vertical grey line in the Time axis). The total O_2 consumed was calculated by subtracting initial and final values of accumulated O_2, which was 48.1 l in this example. The interval between the end of the measurement (468 s) and the recovery of ambient values reflects the estimated time response of the configured system to reach a 95% fractional transformation (~3 min).

3.4. List of symbols and abbreviations

- ATP = Ambient temperature and pressure conditions
- BTPS = Body temperature and water pressure saturated conditions
- EV = Effective volume
- STP = Standard temperature and pressure conditions
- STPD = Standard temperature pressure dry conditions
- T_{exp} = Expiratory duration
- T_{insp} = Inspiratory duration
- T_{tot} = Total breath duration
- $\dot{V}$ = Respiratory flow
- $\dot{V}CO_2$ = Carbon dioxide production rate
- $\dot{V}_{exp}$ = Expiratory flow
- $\dot{V}_{insp}$ = Inspiratory flow
- $\dot{V}O_2$ = Oxygen consumption rate
- V_T = Tidal volume
- V_{Texp} = Expiratory tidal volume
- V_{Tinsp} = Inspiratory tidal volume

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4. Chapter I: Lung function in the Pacific walrus

Lung function assessment in the Pacific walrus (*Odobenus rosmarus divergens*) while resting on land and submerged in water

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Pacific walruses, *Odobenus rosmarus divergens* (Illiger, 1815)
Valencia
Photo credits: Oceanogràfic, Ciudad de las Artes y las Ciencias

4. Chapter I: Lung function in the Pacific walrus

4.1. Abstract

In the present study, lung function in healthy resting adult (born in 2003) Pacific walruses (*Odobenus rosmarus divergens*) was examined by measuring respiratory flow ($\dot{V}$) using a custom-made pneumotachometer. Three female walruses (670 – 1025 kg) voluntarily participated in spirometry trials while spontaneously breathing on land (sitting and lying down in sternal recumbency) and floating in water. While sitting, two walruses performed active respiratory efforts, and one animal participated in lung compliance measurements. For spontaneous breaths, $\dot{V}$ was lower when walruses were lying down (e.g., expiration = 7.1 ± 1.2 l s^{-1}) as compared with in water (9.9 ± 1.4 l s^{-1}), while tidal volume (V_T, 11.5 ± 4.6 l), breath duration (4.6 ± 1.4 s), and respiratory frequency (7.6 ± 2.2 breaths min^{-1}) remained the same. The measured V_T and specific dynamic lung compliance (0.32 ± 0.07 cmH_2O^{-1}) for spontaneous breaths were higher than those estimated for similarly sized terrestrial mammals. V_T increased with body mass (allometric mass-exponent = 1.29) and ranged from 3% to 43% of the estimated total lung capacity (TLC_{est}) for spontaneous breaths. When normalized for TLC_{est}, the maximal expiratory $\dot{V}$ ($\dot{V}_{exp}$) was higher than that estimated in phocids, but lower than that reported in cetaceans and the California sea lion. $\dot{V}_{exp}$ was maintained over all lung volumes during spontaneous and active respiratory manoeuvres. These results suggest that location (water or land) affects lung function in the walrus and should be considered when studying respiratory physiology in semi-aquatic marine mammals.

4.2. Introduction

Marine mammals have to balance available O_2 while using aerobic metabolism during foraging dives and are required to return to the surface to replenish their O_2 stores and remove CO_2. This necessitates efficient gas exchange to rapidly replenish the consumed O_2 before commencing the next dive. The functional and mechanical adaptations of the respiratory system are crucial for gas exchange, and the scientific effort to describe and better understand these features in marine mammals has progressively increased since the first works of Irving (1939) and Scholander (1940). Past work has highlighted the anatomical and functional differences when comparing the respiratory system of marine mammals with that of their terrestrial counterparts (for reviews see: Fahlman et al., 2017; Kooyman, 1973; Piscitelli et al., 2013). For example, studies on the respiratory function in both pinnipeds and cetaceans showed that the tidal volume (V_T, l) and respiratory frequency (f_R, breaths min^{-1}) are, respectively, higher and lower in marine as compared with terrestrial mammals (Fahlman et al., 2017). Also, these studies revealed that, unlike terrestrial mammals, marine mammals are able to generate high respiratory flow ($\dot{V}$, l s^{-1}) during breaths of short duration (Fahlman et al., 2017). In addition, the structure and function of the respiratory system in marine mammals is important in the determination of their diving limitations (Bostrom et al., 2008; Fahlman et al., 2009), and increasing environmental impacts of the ocean (e.g., decreased prey availability or underwater sonar) could lead to an alteration in their diving behaviour. Therefore, an expanded understanding of respiratory function in different marine mammal species is important to gain a better knowledge of the physiological limitations on these species.

The respiratory physiology of the walrus (*Odobenus rosmarus*) has been poorly investigated and data only exist on f_R in wild Atlantic walruses (*O. rosmarus rosmarus*: Bertelsen et al., 2006; Stirling and Sjare, 1988), or f_R and end-expired alveolar CO_2 on one animal under professional care (Mortola and Limoges, 2006; Mortola and Sequin, 2009). Spirometry is a minimally invasive method that provides knowledge on basic respiratory function and mechanics (Burki, 1981; Crapo, 1994), and has recently been adapted for use in voluntarily participating pinnipeds and cetaceans (Fahlman et al., 2020a; Fahlman et al., 2020b; Fahlman et al., 2019a; Fahlman et al., 2019b; Fahlman et al., 2015; Fahlman and Madigan, 2016; Kooyman and Cornell, 1981; Matthews, 1977; Reed et al., 2000). The aim of the present study was to increase basic respiratory physiology data in the walrus by measuring lung function and mechanics in voluntarily

participating adult females using spirometry. A previous study that investigated the California sea lion (*Zalophus californianus*) suggested that the location (resting in water or on land) could affect respiratory function as a result of the pressure on the chest (Fahlman et al., 2020b). Thus, the present study tested the hypothesis that body position on land (sitting or lying down in sternal recumbency) or floating in water, would significantly alter lung function variables (e.g., V_T, f_R, total breath duration [T_{tot}, s], and $\dot{V}$). During the present investigation, it was also hypothesized that expiratory $\dot{V}$ ($\dot{V}_{exp}$) is maintained over most of the vital capacity (VC, l) in the walrus, and that lung compliance ($C_{L,}$ l cmH_2O^{-1}) is higher as compared with that of terrestrial mammals, as previously reported for other marine mammal species (Fahlman et al., 2017).

4.3. Materials and methods

4.3.1. Study subjects

Three adult female Pacific walruses [*Odobenus rosmarus divergens* (Illiger, 1815)], born in 2003 and housed under professional care at the Oceanogràfic (Valencia, Spain), voluntarily participated in the present study. The animals were rescued in the wild as orphan pups and were brought into the aquarium. The health of the walruses was assessed daily, and no pulmonary disease was detected during the data collection period (from February 2015 to July 2018). All experiments were approved by the Animal Care and Welfare Committee of Fundación Oceanogràfic de la Comunitat Valenciana (Valencia, Spain, Animal care number: OCE-19-16) and the U.S. Navy Bureau of Medicine and Surgery (Virginia, USA, BUMED NRD-910).

4.3.2. Experimental procedures, morphometrics and environmental parameters

Spirometry trials while spontaneously breathing were performed while inactive in three different body positions: 1) lying down in sternal recumbency, 2) sitting while supported by the pectoral flippers and 3) floating on the water surface (see Annex A for an example of similar body positions while measuring lung function in California sea lions in Fahlman et al., 2020b). The walruses adopted a vertical position while floating in a 3 m deep seawater pool. The estimated water height acting on the centroid of the lungs was approximately 40 cm. For experimental trials while spontaneously breathing, only those trials where the walruses were resting for at least 2 minutes while performing complete breaths (composed of an expiration and an inspiration) were included in the analysis. Two of the walruses were trained to perform 5-10 consecutive maximal

respiratory efforts while sitting on land. The animals performed these manoeuvres at their individual capacity and will be referred as active respiratory efforts/manoeuvres, where the maximum measured V_T was considered as the behavioural vital capacity (VC_B, l). In addition, one walrus was trained to swallow an oesophageal balloon catheter that allowed measurement of the dynamic C_L during spontaneous breaths while sitting. Body mass (M_b, kg) of each animal was recorded in the same week as lung function testing. For all experimental trials, the ambient pressure, and air temperature and humidity (thermometer and hygrometer OH513 Oh Haus & Co.) were measured prior to the spirometry trial. The average (± standard deviation, s.d.) water temperature at the facility housing the animals during the experimental period was 15.9 ± 0.9°C (range = 14.5 – 20.1°C).

4.3.3. Respiratory flow measurements

$\dot{V}$ was measured using a custom-made Fleisch type pneumotachometer (Mellow Design, Valencia, Spain) with a soft silicone ring at the base and a dead space of 700 ml (see Figures 3.1 and 3.2 in the General methodology section). A low-resistance laminar flow matrix (Item #Z9A887-2, Merriam Process Technologies, Cleveland, OH, USA) was placed inside the pneumotachometer to create the pressure difference during respiratory data collection, which was measured with a differential pressure transducer (Spirometer Pod, ML 311, ADInstruments, Colorado Springs, CO, USA). This differential pressure transducer was connected to the pneumotachometer via two firm-walled, flexible tubes of 310 cm length and 2 mm internal diameter (i.d.). The pneumotachometer was placed over the snout and gently pressed down to prevent leaks around the silicone base while collecting respiratory data. The walrus was trained to close its mouth during data collection to ensure respiration only through the nostrils, and the trainer positioned one hand over the mouth, which allowed any possible leaks to be detected (see Figure 3.1 in the General methodology section). The pneumotachometer was calibrated for linearity between flow and resistance using a 7 l calibration syringe (Series 4900, Hans-Rudolph Inc., Shawnee, KS, USA) following detailed procedures in the General methodology section of this thesis.

4.3.4. Airway and oesophageal pressure measurements

For estimating the dynamic C_L, the oesophageal (P_{oeso}, cmH_2O) and airway opening pressures (P_{ao}, cmH_2O) were measured during spontaneous breaths (Fahlman et al., 2015; Olsen et al., 1969). An oesophageal balloon catheter (47-9005, Cooper Surgical, Trumbull, CT, USA) was manually inserted into the oesophagus for measuring the P_{oeso},

and a sample port was placed above the nostrils of the walrus for measuring the P_{ao}. The catheter was placed at the level of the heart and inflated with 1.0 ml of air. Both sample lines were connected to a differential pressure transducer (MPX-100 mbar type 339/2, Harvard apparatus, Holliston, MA, USA) through 288 cm length and 2 mm i.d., firm-walled and flexible tubes.

4.3.5. Data acquisition and processing

Measured differential pressures were captured at 400 Hz using a data acquisition system (Powerlab 8/35, ADInstruments) and displayed on a laptop computer running LabChart (v. 8.1, ADInstruments).

Calibrated and filtered inspiratory $\dot{V}$ ($\dot{V}_{insp}$) and $\dot{V}_{exp}$ were integrated to estimate inspiratory (V_{Tinsp}) and expiratory (V_{Texp}) tidal volume, which were converted into standard temperature and pressure dry (STPD, Quanjer et al., 1993), as previously detailed in the General methodology section of this thesis. f_R was calculated for each trial using the number of complete breaths divided by the measurement period.

The dynamic C_L was estimated as the V_{Tinsp} divided by the tidal change in transpulmonary pressure (P_L, $cmH_2O = P_{ao} - P_{oeso}$) measured at zero flow, following previous procedures (see Annex B from Fahlman and Madigan, 2016). The reference pressure for both P_{ao} and P_{oeso} was the ambient atmospheric pressure (P_{amb}). As C_L varies with lung size (Stahl, 1967), the specific C_L (sC_L, cmH_2O^{-1}) was computed by dividing C_L by the minimum air volume (MAV, l) or the volume of air left in the relaxed lung, which was estimated to be 7% of total lung capacity (TLC, l), based on previous experiments with excised lungs (Fahlman et al., 2011).

4.3.6. Statistical analysis

For the statistical analysis of spontaneous breaths, only periods of normal and complete breaths were considered, and single expirations or inspirations were removed as in previous studies (Fahlman and Madigan, 2016; Fahlman et al., 2020b). For analysis of active breaths, only the 2-4 largest and most similar manoeuvres for each trial were included. The difference between expiratory and inspiratory $\dot{V}$, V_T and duration for spontaneous (paired *t*-test) and active breaths (Wilcoxon signed-rank test) were analysed using the Statistical Product and Service Solutions (SPSS, v.24.0, released 2016. IBM SPSS Statistics for Windows, IBM Corp., Armonk, NY, USA). The relationship between measured dependent variables ($\dot{V}$, V_T, and f_R) while spontaneously breathing and M_b was

analysed using linear mixed-effects models using the lme function in R (R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, v.3.6.1, 2019). The individual animal was treated as a random effect, which accounted for the correlation between repeated measurements on the same individual (Littell et al., 1998). Homoscedasticity for all models was confirmed by the Bartlett test, and in case of unequal variances the variable was $\log_{10}$-transformed. Best models of remaining variables were chosen by the log-likelihood (LL) ratio test. The effect of the experimental factor (body position) on measured dependent variables ($\dot{V}$, V_T, T_{tot} and f_R) for spontaneous breaths was analysed using a two-way Mixed-Effects ANOVA (SPSS), where the body position was treated as a fixed effect and individual animal as a random effect (Frederick, 1999). In this study p-values ≤ 0.05 were considered as significant, and data are presented as means ± s.d.

4.4. Results

Data were collected from a total of 120 trials performed from February 2015 to July 2018 (2015: n = 25, 2016: n = 1, 2017: n = 52, 2018: n = 42), and a subset of 5 trials for each animal and body position (15 trials per position, n = 45) were selected for the lung function analysis. In addition, a total of 9 separate trials while sitting included active respiratory manoeuvres, and 3 separate trials measured dynamic C_L. The breathing pattern in the walrus began with an expiration followed by an inspiration and an end-inspiratory pause. For lung function measurements from the three walruses while they were resting and spontaneously breathing in all body positions, the average trial duration was 3.9 ± 1.1 min (range = 2.0-7.2 min, n = 45) with a total of 763 complete breaths (Table 4.1). For separate trials while walruses were sitting, a total of 26 active respiratory manoeuvres from two animals were included in the analysis (Table 4.1), and 21 spontaneous breaths were used to estimate the dynamic C_L in one individual. For the trials included in the study, the ambient pressure was 101.4 ± 0.5 kPa (range = 99.2 – 102.4 kPa), while the air temperature and humidity were 21.3 ± 3.1°C (16.0 – 27.6°C) and 74.1 ± 8.3% (57.0 – 99.0%), respectively.

Table 4.1. Trial and sample details of lung function measurements in Pacific walruses.

Animal ID	Nº trials	Nº spontaneous breaths	Nº active breaths	Trial duration (min)
26005388	38	237	16	3.4 ± 0.8
26005389	39	288	10	4.0 ± 1.0
26005390	43	238	-	4.3 ± 1.4

Animal identification (ID) of three voluntarily participating adult (birth year 2003) female Pacific walruses (*Odobenus rosmarus divergens*), total number of trials (including training sessions and analysed trials), total number of analysed spontaneous and active breaths, and mean (± s.d., n = 45) total trial duration.

4.4.1. Inspiratory and expiratory phases

For each animal, the mean T_{tot}, expiratory (T_{exp}) and inspiratory (T_{insp}) duration, $\dot{V}_{exp}$, $\dot{V}_{insp}$, V_{Texp} and V_{Tinsp} for spontaneous and active breaths are reported, respectively, in Table 4.2 and Table 4.3. For spontaneous breaths, the average T_{tot} was 4.6 ± 1.4 s, where T_{exp} was significantly shorter (2.1 ± 0.7 s) than T_{insp} (2.5 ± 0.9 s, paired *t* test, *t* value = -16.2, df = 762, $p < 0.01$). Mean $\dot{V}_{exp}$ was significantly higher (8.5 ± 2.8 l s^{-1}) than $\dot{V}_{insp}$ (6.4 ± 1.9 l s^{-1}, *t* value = 25.3, df = 762, $p < 0.01$). V_T did not differ between expiration (11.5 ± 4.6 l) and inspiration (11.7 ± 4.6 l, *t* value = -1.74, df = 762, $p > 0.05$), and therefore, only V_{Texp} is reported when referring to V_T for spontaneous breaths, unless otherwise specified. For active respiratory manoeuvres, average T_{tot} was 3.6 ± 0.9 s, where T_{exp} was significantly shorter (0.9 ± 0.3 s) than T_{insp} (2.7 ± 0.8 s, Wilcoxon signed-rank test, T = 351, $p < 0.01$), while average $\dot{V}_{exp}$ was significantly higher (35.9 ± 10.0 l s^{-1}) than $\dot{V}_{insp}$ (10.3 ± 0.8 l s^{-1}, T = 0, $p < 0.01$). There were no differences between V_{Texp} (18.8 ± 5.7 l) and V_{Tinsp} (19.8 ± 5.4 l, T = 194, $p > 0.5$) for active respiratory manoeuvres.

Table 4.2. Lung function results for spontaneous breaths in Pacific walruses.

Animal ID	$\dot{V}$ (l s^{-1})		V_T (l)		T (s)		
	Expiratory	Inspiratory	Expiratory	Inspiratory	Expiratory	Inspiratory	Total
26005388	9.1 ± 2.5 (2.9-16.7)	5.6 ± 1.5 (2.4-9.2)	9.6 ± 3.0 (2.6-17.0)	9.7 ± 2.8 (3.4-17.7)	1.7 ± 0.6	2.5 ± 0.8	4.2 ± 1.3
26005389	9.0 ± 2.6 (3.1-19.5)	7.0 ± 1.8 (1.9-11.8)	12.7 ± 5.0 (3.4-28.6)	12.8 ± 5.1 (3.5-28.8)	2.0 ± 0.6	2.4 ± 0.9	4.4 ± 1.2
26005390	7.1 ± 2.8 (1.7-16.0)	6.5 ± 2.0 (2.8-14.7)	12.0 ± 4.9 (1.8-27.1)	12.2 ± 4.9 (2.2-26.9)	2.5 ± 0.7	2.7 ± 0.9	5.2 ± 1.4

Animal ID, mean (± s.d., n = 763) and range for all body positions (sitting supported by the front flippers, lying down, and floating at rest in water) of expiratory and inspiratory flow ($\dot{V}$), tidal volume (V_T), and average duration (T) for each respiratory phase and total breath duration obtained from three voluntarily participating adult female Pacific walruses (*O. rosmarus divergens*) while spontaneously breathing.

Table 4.3. Lung function results for active breaths in Pacific walruses.

Animal ID	$\dot{V}$ (l s^{-1})		V_T (l)		T (s)		
	Expiratory	Inspiratory	Expiratory	Inspiratory	Expiratory	Inspiratory	Total
26005388	29.2 ± 5.3 (21.9-38.3)	10.2 ± 1.4 (6.7-11.4)	19.4 ± 4.0 (9.8-25.0)	19.6 ± 3.4 (13.5-24.6)	1.0 ± 0.2	2.9 ± 0.8	3.9 ± 0.9
26005390	46.6 ± 5.2 (39.5-55.4)	11.3 ± 2.0 (9.4-16.0)	17.8 ± 8.0 (10.1-28.9)	20.2 ± 7.8 (10.7-31.9)	0.6 ± 0.2	2.5 ± 0.7	3.1 ± 0.8

Animal ID, mean (± s.d. n = 26) and range of expiratory and inspiratory flow ($\dot{V}$), tidal volume (V_T), and average duration (T) for each respiratory phase and total breath duration of active respiratory manoeuvres obtained from two voluntarily participating adult female Pacific walruses (*O. rosmarus divergens*) while sitting and supported by their pectoral flippers.

4.4.2. Study of body position

For the 45 selected trials to assess lung function from spontaneous breaths, T_{tot} did not significantly change with the body position of the animals (sitting = 5.4 ± 1.3 s; lying down = 4.9 ± 0.8 s; water = 3.9 ± 0.8 s, two-way Mixed-Effects ANOVA, F [2, 4] = 3.92, $p > 0.1$, Figure 4.1). The body position of the animals significantly affected $\dot{V}$ (expiration: F [2, 4] = 18.60, $p < 0.01$; inspiration: F [2, 4] = 10.42, $p < 0.05$), where both $\dot{V}_{exp}$ and $\dot{V}_{insp}$ were significantly lower when lying down (expiration = 7.1 ± 1.2 l s^{-1}; inspiration = 5.5 ± 1.1 l s^{-1}) as compared with when in water (expiration = 9.9 ± 1.4 l s^{-1}; inspiration = 7.2 ± 1.2 l s^{-1}, Tukey *post-hoc* test, $p < 0.001$ for all, Figure 4.1). The mass-specific V_T (sV_T, ml kg^{-1}) did not significantly differ between the three body positions (sitting = 15.7 ± 2.6 ml kg^{-1}; lying down = 13.3 ± 2.5 ml kg^{-1}; water = 13.8 ± 2.8 ml kg^{-1}, F [2, 4] = 2.7, $p > 0.1$). Similarly, no differences were detected between body positions for f_R (sitting = 6.2 ± 2.3 breaths min^{-1}; lying down = 8.3 ± 1.7 breaths min^{-1}; water = 8.4 ± 2.0 breaths min^{-1}, F [2, 4] = 6.07, $p > 0.05$).

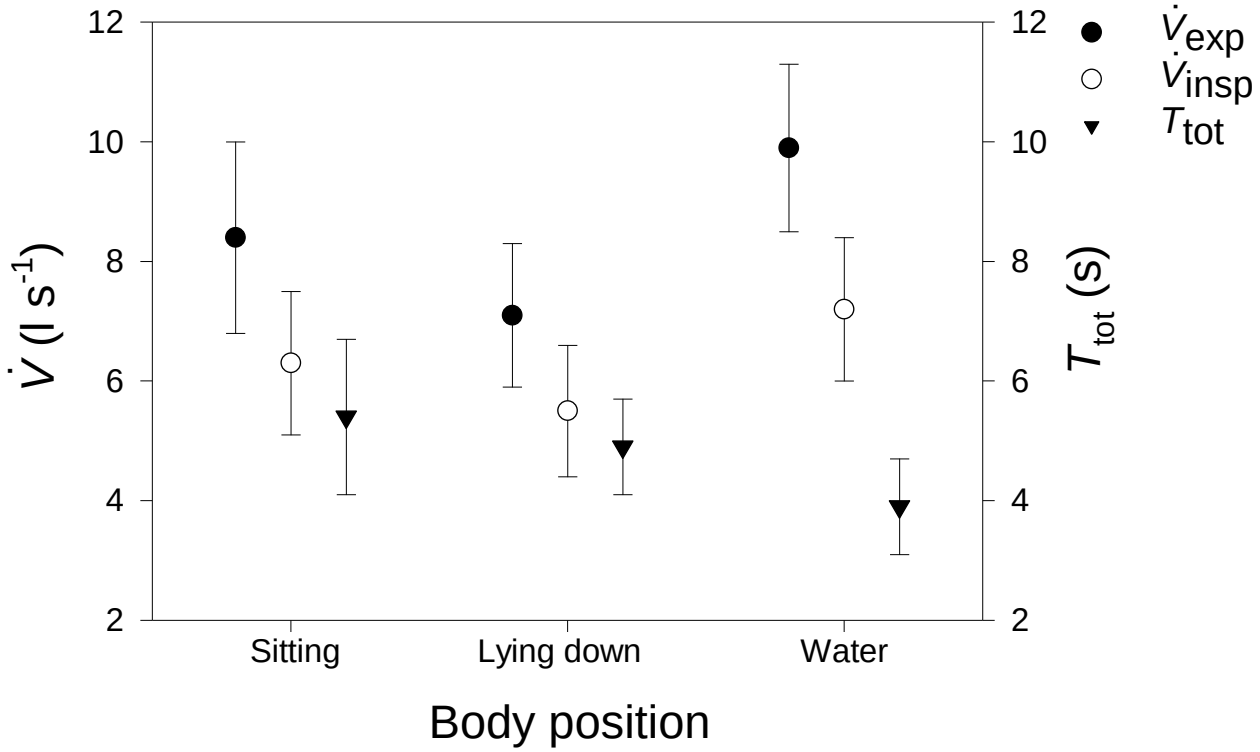

Figure 4.1. Measured respiratory variables from Pacific walruses while spontaneously breathing in different body positions.
Average (± s.d., n = 15 for each position) respiratory flow ($\dot{V}$) during expiration ($\dot{V}_{exp}$) and inspiration ($\dot{V}_{insp}$), and total breath duration (T_{tot}) measured from three voluntarily participating adult female Pacific walruses (*Odobenus rosmarus divergens*) while sitting and supported by the front flippers (sitting), lying down in sternal recoumbency (lying down) and in water (water).

4.4.3. General morphometrics, lung function, and dynamic lung compliance

For trials included in the analysis the M_b averaged 822 ± 96 kg (range = 670 – 1025 kg, Table 4.4). For all body positions and spontaneous breaths, the average f_R for all trials was 7.6 ± 2.2 breaths min^{-1} (Table 4.4). The average sV_T while spontaneously breathing was 13.9 ± 5.2 ml kg^{-1} (range = 2.2-32.7 ml kg^{-1}), and the highest $\dot{V}$ (19.5 l s^{-1}) and the largest V_T (28.8 l, sV_T = 32.7 ml kg^{-1}) were measured while floating at the water surface (Animal ID: 26005389, Table 4.2). TLC_{est} was calculated based on data from excised lungs of different species of marine mammals, including juvenile walruses ($TLC_{est} = 0.135 \cdot M_b^{0.92}$, where M_b is in kg and TLC_{est} is in l: Kooyman, 1973), and using the M_b measured during the experimental period (Table 4.4). The average TLC_{est} was 63.2 ± 8.3 l (Table 4.4) and for the three body positions and spontaneous breaths, the V_T ranged from 3% to 43% of the TLC_{est}. When performing active respiratory manoeuvres while sitting, the maximal $\dot{V}$ (55.4 l s^{-1}) and the largest V_T (31.9 l) were measured for walrus 26005390 (Table 4.3), and the V_T reached 50% of TLC_{est}.

Table 4.4. Body mass, estimated total lung capacity, and respiratory frequency of Pacific walruses.

Animal ID	$M_{b\text{-}LF}$ (kg)	$M_{b\text{-}Exp}$ (kg)	TLC_{est} (l)	f_R (breaths min^{-1})
26005388	715 ± 17	674 ± 45	54.0 ± 3.3	7.9 ± 1.9 (5.5-12.2)
26005389	931 ± 43	917 ± 76	71.7 ± 5.4	8.4 ± 2.0 (4.2-10.7)
26005390	821 ± 38	807 ± 44	63.7 ± 3.2	6.6 ± 2.5 (3.1-10.8)

Animal ID of three voluntarily participating adult female Pacific walruses (*O. rosmarus divergens*), mean (± s.d.) body mass ($M_{b\text{-}LF}$) measured the same week as lung function testing (± 0-4 days), body mass measured during the experimental period ($M_{b\text{-}Exp}$), estimated total lung capacity (TLC_{est}) based on a previous allometric equation for marine mammals ($TLC_{est} = 0.135 \cdot M_b^{0.92}$, where M_b is in kg and TLC_{est} in l: Kooyman, 1973), and average and range for measured respiratory frequency (f_R) during the experimental procedure.

For spontaneous breaths, there was a positive correlation between V_{Texp} and V_{Tinsp} and M_b with a mass-exponent close to unity (Figure 4.2, Table 4.5). During spontaneous breathing, neither $\dot{V}_{exp}$ nor $\dot{V}_{insp}$ correlated with M_b (Table 4.5). There was a positive correlation between f_R and M_b (Table 4.5).

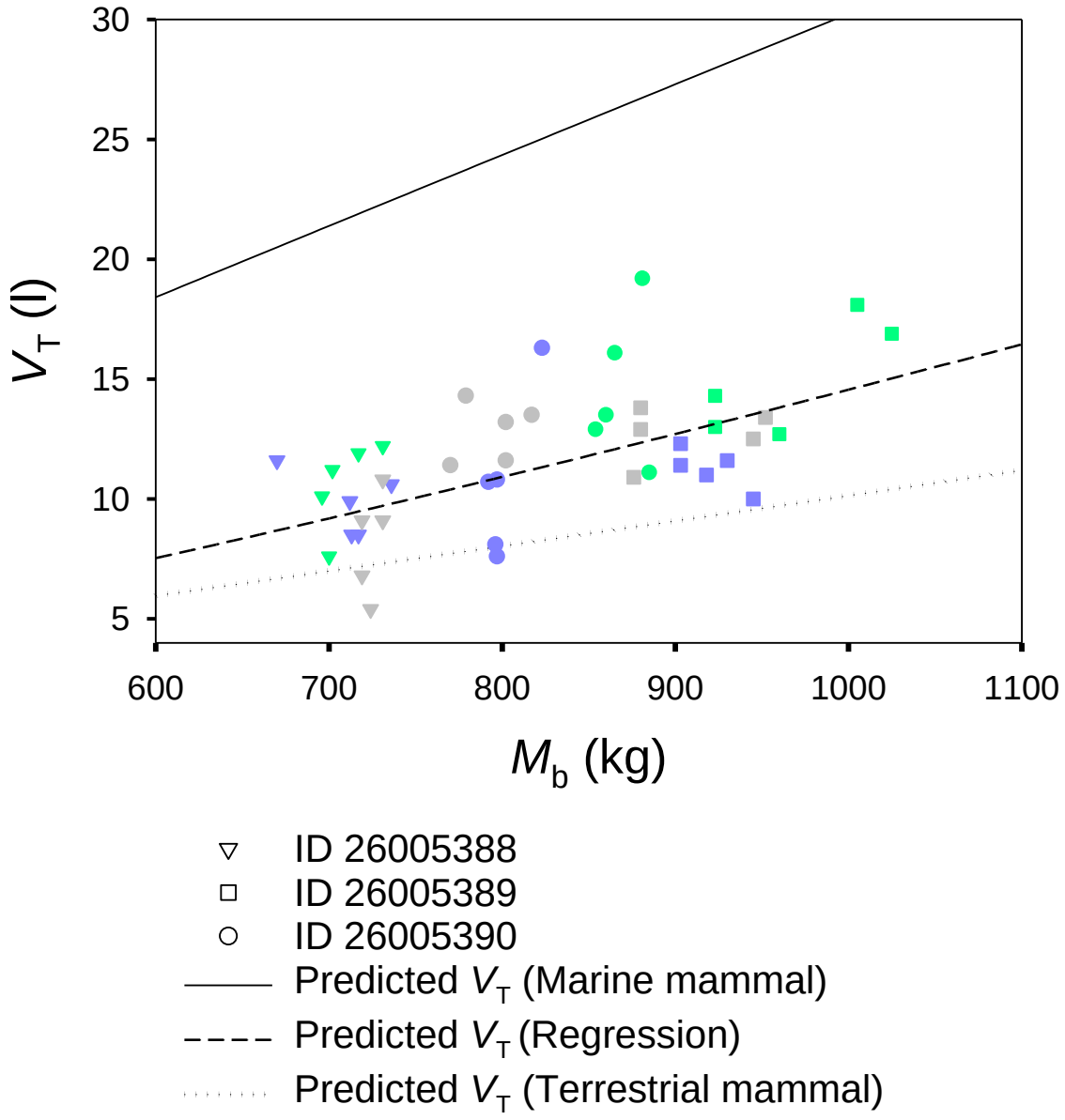

Figure 4.2. Relationship between body mass and tidal volume during spontaneous breathing in Pacific walruses.
Measured body mass (M_b) and tidal volume (V_T) from three voluntarily participating adult female Pacific walruses (*O. rosmarus divergens*) in three different body positions: sitting supported by the pectoral flippers (green filled symbols), lying down (blue) and floating in water (grey). The dashed line represents the regression line based in obtained results in Table 4.5 for expiratory tidal volume (V_{Texp}). The solid line shows the predicted V_T using the allometric equation obtained from a number of marine mammal species ($V_T = 0.0372 \cdot M_b^{0.97}$: Fahlman et al., 2020b), while the dotted line represents the estimated V_T for terrestrial mammals at rest ($V_T = 7.69 \cdot M_b^{1.04}$: Stahl, 1967).

Table 4.5. Statistical results evaluating the relationship between respiratory variables and body mass for voluntary breaths in Pacific walruses.

Dependent	χ^2	*p*-value	β_0	$\log_{10}(M_b)$
$\log_{10}(V_{Texp})$	10.8	<0.01	-2.72 ± 0.80	1.29 ± 0.27
$\log_{10}(V_{Tinsp})$	10.4	<0.01	-2.54 ± 0.82	1.24 ± 0.28
$\dot{V}_{exp}$	<0.01	=0.99	----	----
$\dot{V}_{insp}$	2.5	>0.1	----	----
$\log_{10}(f_R)$	4.2	<0.05	9.20 ± 2.78	-2.86 ± 0.96

Linear mixed-effect models including expiratory (exp) and inspiratory (insp) tidal volume (V_T, l), respiratory flow ($\dot{V}$, l s^{-1}), and respiratory frequency (f_R, breaths min^{-1}) measured for all body positions (sitting supported by the front flippers, lying down, and floating at rest in water) and spontaneous breaths in three adult female Pacific walruses (*O. rosmarus divergens*). V_T, f_R and body mass (M_b) were transformed using the base 10 logarithm ($\log_{10}$). The results show the χ^2 for the log-likelihood ratio test and the obtained p-value when comparing the model including M_b with the model including only the intercept. The intercept (β_0), and the coefficient for $\log_{10}(M_b)$ together with the standard error resulting from the final models including M_b are additionally reported.

The average dynamic C_L measured in one walrus (Animal ID: 26005388) while spontaneously breathing was 1.09 ± 0.23 l cmH_2O^{-1}. The M_b of the walrus 26005388 at the time of the measurements was 640 kg, resulting in an average dynamic sC_L of 0.32 ± 0.07 cmH_2O^{-1}.

The range of $\dot{V}_{exp}$ and $\dot{V}_{insp}$ for spontaneous and active breaths combined was 1.7-55.4 l s^{-1} and 1.9-16.0 l s^{-1}, respectively (Table 4.2 and Table 4.3). When $\dot{V}_{exp}$ and $\dot{V}_{insp}$ were normalized to TLC_{est} (specific respiratory flow: $s\dot{V}$, s^{-1}), the $s\dot{V}$ for expiration ($s\dot{V}_{exp}$) ranged from 0.03 to 0.87 s^{-1} and that for inspiration ($s\dot{V}_{insp}$) ranged from 0.03 to 0.25 s^{-1}. The flow-volume relationship for two animals while sitting showed that $\dot{V}_{exp}$ and $\dot{V}_{insp}$ were constant over all lung volumes during both, spontaneous and active breaths (Figure 4.3).

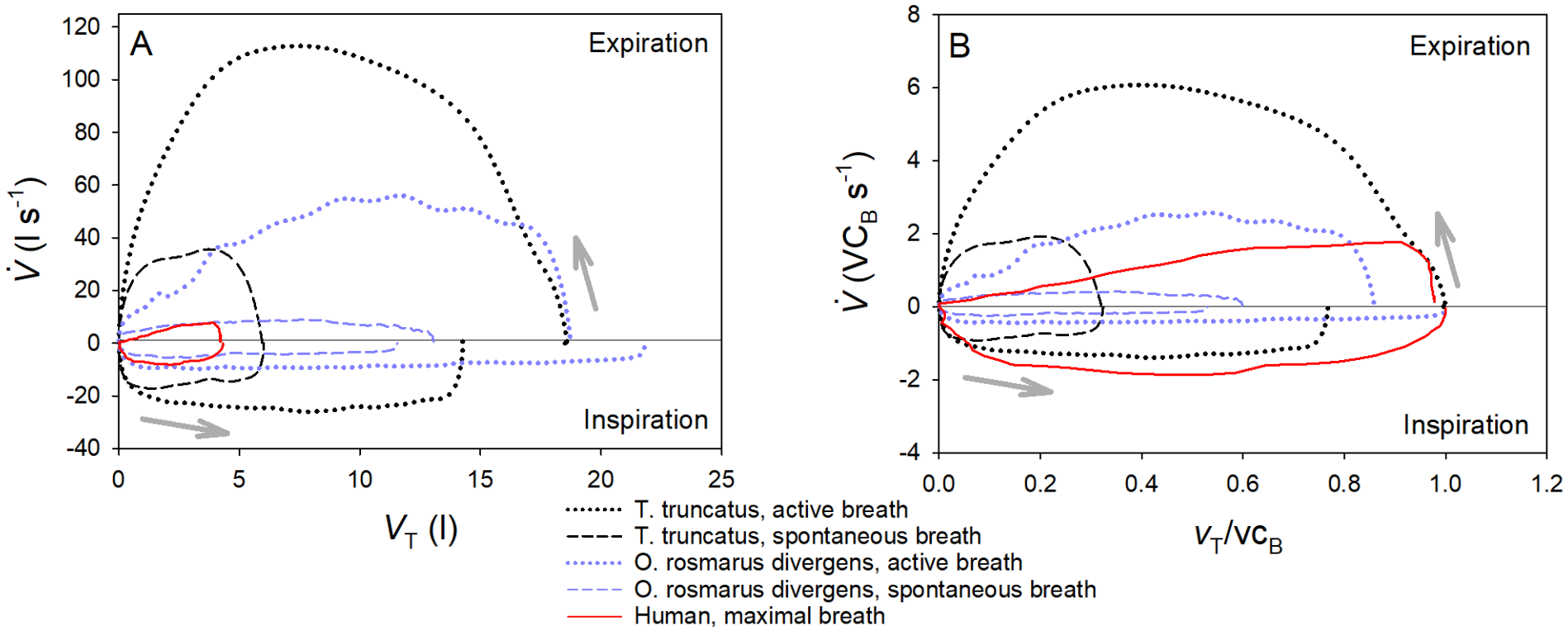

Figure 4.3. Comparative flow-volume relationship for Pacific walrus, bottlenose dolphin and human.

Data from one adult female Pacific walrus (*O. rosmarus divergens*) in the present study (animal ID = 26005390, year of birth 2003, 821 ± 38 kg), one adult male bottlenose dolphin *(Tursiops truncatus*, age 21 years, 197 kg; Fahlman et al., 2015), and one adult man (Figure 4 of Miller et al., 2005; reproduced with permission of the ERS © 2020: European Respiratory Journal 2005 26: 319-338; DOI: 10.1183/09031936.05.00034805). A) Flow-volume relationship for spontaneous and active respiratory manoeuvres from both species of marine mammals, and for one maximal respiratory manoeuvre from the adult human male. B) For the same data, respiratory flow ($\dot{V}$) and tidal volume (V_T) were normalized by the maximum measured V_T for the represented active manoeuvre (walrus = 21.8 l, dolphin = 18.6 l, human = 4.3 l), which was considered as the behavioural vital capacity (VC_B). For each representation, the arrow on the right shows the direction of the expiratory phase at the beginning of the respiratory manoeuvres (positive values of respiratory flow or normalized respiratory flow), while the arrow on the left shows the inspiratory phase (negative values). Spontaneous and active breaths were collected through voluntary participation of the animals, where the walrus was sitting and supported by the pectoral flippers and the dolphin was floating in water.

4.5. Discussion

The respiratory variables collected in the present study were within the previous ranges measured for other marine mammal species and differed from that reported for terrestrial mammals (see a comparative summary in Table 4.6). The walruses showed a lower f_R than that expected for a similarly sized terrestrial mammal. The breathing pattern in the walruses was similar to that reported in other pinnipeds (Kooyman, 1973), and began with an expiration, followed by an inspiration and a respiratory pause. Obtained results in the present study showed that $\dot{V}$ was lower while lying down than when floating in water, while sV_T, T_{tot} and f_R remained the same for all body positions. Measured V_T was higher than that estimated for a terrestrial mammal of similar size (Stahl, 1967), increased with M_b with a mass-exponent close to unity, and reached 50% of TCL_{est} during active respiratory manoeuvres. Measured dynamic sC_L in one walrus was higher than that reported for terrestrial mammals, and was similar to that previously measured in pinnipeds and cetaceans (Fahlman et al., 2017). The peak s$\dot{V}_{exp}$ for active breaths was lower than that reported for cetaceans and California sea lions (Kerem et al., 1975), but higher than that estimated for Weddell and grey seals (Falke et al., 2008; Reed at al., 1994). The flow-volume relationship showed that both $\dot{V}_{exp}$ and $\dot{V}_{insp}$ are maintained over the entire lung volume when performing both spontaneous and active respiratory manoeuvres.

Studies on respiratory function and mechanics in marine mammals have used different approaches. Some studies have used anesthetized or post-mortem animals and excised tissues (Denison and Kooyman, 1973; Denk et al., 2020; Fahlman et al., 2011; Fahlman et al., 2014; Kooyman and Sinnett, 1979; Leith et al., 1972; Moore et al., 2011), which may not reflect respiratory function in a realistic biological scenario (Fahlman et al., 2017). Other studies have used restrained and/or involuntarily participating animals (Falke et al., 2008; Gallivan, 1981; Irving et al., 1941; Kerem et al., 1975; Kooyman et al., 1971; Olsen et al., 1969; Reed et al., 1994; Scholander and Irving, 1941; Spencer et al., 1967; Wahrenbrock et al., 1974), probably resulting in stress, which may affect the physiology and breathing patterns. As one important aspect when studying physiology is to minimize confounding variables during data collection, studies on trained animals may help minimize stress during voluntary participation (Fahlman et al., 2017). However, one disadvantage when working with trained animals is that voluntary compliance may differ between days.

Table 4.7. Summary of normalized respiratory variables for a number of resting marine mammals.

Species	N	Stage	M_b (kg)	TLC_{est} (l)	sV_T (ml kg^{-1})	$\%TLC_{est}$ (%)	sC_L (cmH_2O^{-1})	C_L/TLC_{est} (cmH_2O^{-1})	$s\dot{V}_E$ (l min^{-1} kg^{-1})	$\dot{V}_E/TLC_{est}$ (min^{-1})	$s\dot{V}_{exp}$ (s^{-1})
[a, b]Human		A	70	4.8	9.1	14	0.07	0.04	0.17	2.5	2.3
[c]Walrus	3	A	596-1064*	63.2 ± 8.3	13.9 ± 5.2	3-43 (50)	0.32 ± 0.07[1]	0.02[1]	0.11 ± 0.03	1.3 ± 0.34	0.86
[d]Grey seal‡‡	4	A	140-250	17.3	32.3	35-49			0.63	7.1	0.57‡
[e]Weddell seal	3	Sa	260-345**	24.8	17.0	20 (46‡‡)			0.11	1.3	0.29‡‡
[f, g, h]California sea lion	13	A & J	49-130	7.4	25.1 ± 1.7	26-30 (86§)			0.16	1.7	~3.9§
	4+	A & J	17-126	5.5 ± 4.2			0.92 ± 0.16[4]	0.07[4]			
[i]Patagonia sea lion	5	A	94-286	13.4	28.5	47-73	0.41 ± 0.11[4]	0.03[4]	0.13	1.4	
[j]Bottlenose dolphin	6	A	158-250	16.8	30.8	34 (106)	0.31 ± 0.04[6]	0.02[6]	0.10	1.2	8.0
[k]Beluga	9	A	510-891**	54.6	25.5	14-34 (97)			0.11	1.4	4.7

Common name (Species), number of studied individuals (N), reproductive stage (Stage; A = adults, Sa = subadults, and J = juveniles), measured body mass (M_b) range, and estimated total lung capacity ($TLC_{est} = 0.135 \cdot M_b^{0.92}$: Kooyman, 1973) from previous studies in marine mammals. Superscripts letters in the common name column indicate the reference from which the data is reported. Estimated values for an adult male human (M_b = 70 kg, height = 175 cm, age = 40 years) are reported as a representation of terrestrial mammals. Listed values or ranges have been estimated from previous measured variables, while measured ranges or means (± s.d.) are provided when available. For tidal volume (V_T) and respiratory flow ($\dot{V}$), data for the expiratory phase is preferentially reported when available. For spontaneous breaths while resting: mass-specific tidal volume (sV_T), percentage of TLC_{est} reached ($\%TLC_{est}$), specific lung compliance (sC_L; lung compliance divided by the minimum air volume, MAV), lung compliance normalized by TLC_{est} (C_L/TLC_{est}), mass-specific respiratory minute volume ($s\dot{V}_E$), and respiratory minute volume ($\dot{V}_E$) normalized by TLC_{est} ($\dot{V}_E/TLC_{est}$). For collected data from respiratory efforts, the estimated $\%TLC_{est}$ (values in parentheses), and the specific expiratory flow ($s\dot{V}_{exp}$) normalized by the TLC_{est} for the reported maximal respiratory manoeuvre are indicated. [a]Radeos and Camargo (2004); [b]Stahl (1967); [c]Present study; [d]Reed et al., (1994); [e]Falke et al., (2008); [f]Fahlman et al., (2020b); [g]Kerem et al., (1975); [h]Fahlman et al., (2014); [i]Fahlman and Madigan (2016); [j]Fahlman et al., (2015); [k]Fahlman et al., (2019b). *Range of measured M_b through the entire experimental period in the present study. Superscripts numbers indicate the number of individuals that underwent lung compliance measurements. **All or some M_b values were estimated. ‡Estimated from the average for maximal reported manoeuvres. ‡‡Data collected during surface periods after breath-holds. §Measured from a 2 year old, 32 kg subject from Kerem et al., (1975). +Respiratory data collected from anesthetized individuals in Fahlman et al., (2014).

Also, the influence of the trainer may alter the breathing pattern (e.g., anticipatory behaviour could increase respiratory frequency), causing a bias that has to be assessed. For this reason, the subjects should undergo desensitization to the experimental procedures to ensure that data collection is minimally affected. In addition, it is also important to critically evaluate the data and assess whether the measurement has influenced the subject.

4.5.1. General physiological state of the study subjects

In the present study, the walruses participated in a total of 120 trials to ensure desensitization to the lung function procedure. A subset of these trials where the animals were calm and breathing normally was analysed and reported. Measured f_R during the experiments in the present study (7.6 ± 2.2 breaths min^{-1}) was higher than that estimated in resting semi-aquatic and fully aquatic mammals ($f_R = 33 \cdot M_b^{-0.42}$: Mortola and Limoges, 2006), but lower than that predicted for terrestrial mammals of the same size ($f_R = 53.5 \cdot M_b^{-0.26}$: Stahl, 1967). These measurements were close to data obtained from the same animals while floating inside a respirometer (5.8 ± 2.3 breaths min^{-1}: see Table S1 in Chapter III in the present thesis), and to those reported in a single walrus under human care (5.9 breaths min^{-1}: Mortola and Sequin, 2009).

The respiratory minute volume ($\dot{V}_E = f_R \cdot V_T$, l min^{-1}) is commonly used as a measure of the volume of air that is moved in and out of the lungs, and provides an alternative method to evaluate departure from normal ventilatory patterns and whether an increase in f_R results in hyperventilation (Prakash, 2015). For the three walruses participating in the present study, similar results were found when comparing the average mass-specific respiratory minute volume ($s\dot{V}_E$, l min^{-1} kg^{-1}) during expiration, with estimated $s\dot{V}_E$ calculated from previous reported data in resting trained marine mammals that were additionally monitored through focal observations (Table 4.6). Thus, the estimated $s\dot{V}_E$ suggests that the walruses were not hyperventilating but may have increased f_R during some experimental trials (see f_R ranges in Table 4.4), while at the same time reducing V_T to retain a normal $\dot{V}_E$ and alveolar minute ventilation. This could also explain the wide range of measured V_T from spontaneous breaths (3-43% of TLC_{est}) when compared with previous studies in marine mammals (Table 4.6), and the obtained mass-exponent for f_R (Table 4.5) when compared with previous allometric equations (Fahlman et al., 2020a; Mortola and Limoges, 2006). The variability in the reported f_R could suggest a sign of anticipatory behaviour caused by unintentional conditioning, as

the animals were positively reinforced for complete breaths during the desensitization period. Despite this, the average and ranges for respiratory variables in the present study agree with those previously reported for marine mammals. Thus, although working with trained animals could result in a bias that should be considered and critically evaluated, the use of animals in managed care allows for data collection in a controlled environment where the animal welfare is a priority. In addition, voluntary participation of trained animals provides a manageable opportunity to obtain measurements that may be difficult and/or ethically challenging to collect from wild megafauna.

4.5.2. The effect of body position

Walruses, like other semi-aquatic species, spend part of their time on land and their respiratory function should be adapted to the two different media. When pinnipeds are lying on land, gravity could be affecting lung function as a result of increased pressure on the thoracic cage (Fahlman and Madigan, 2016). Similarly, the hydrostatic pressure of the water column on the chest could also affect lung function when floating at the water surface, as recently suggested for California sea lions (Fahlman et al., 2020b). Therefore, lung function in water and on land (sitting and lying) was tested to assess changes in lung function in the Pacific walrus. Obtained sV_T, T_{tot}, and f_R remained the same for the three body positions, whereas measured $\dot{V}$ was higher when the animals were floating in water as compared to when lying down. Thus, obtained $\dot{V}_{exp}$ and $\dot{V}_{insp}$ are similar to those previously reported for California sea lions where it was suggested that the increased hydrostatic pressure on the chest helped to increase $\dot{V}$, while T_{tot} decreased to achieve the same V_T and alveolar ventilation as on land (Fahlman et al., 2020b). While in the present study T_{tot} did not change with body position, the higher $\dot{V}$ obtained when floating in water agrees with the previously suggested pressure effect of the water column.

In marine mammals, the expiratory phase is passive and mainly driven by the elastic recoil of the chest, while the inspiratory phase is active (Fahlman et al., 2017), and respiratory function could be limited by the gravity and the increased pressure on the chest when lying on land. Indeed, the reported $\dot{V}$ while lying on land in the present study was lower than when floating in water, which could suggest a possible flow limitation on land. However, the hydrostatic pressure could help assist elastic recoil during the expiration, allowing for a passive increase in the $\dot{V}_{exp}$ while floating in water, as previously suggested (Fahlman et al., 2020b). While the results of the present study do not provide sufficient evidence of a gravitational alleviation while sitting as compared to

lying, this possible effect should not be discarded. Therefore, further studies on semi-aquatic species, would help confirm the respiratory flow-volume limitations related to the body position and the media location in these species.

4.5.3. Tidal volume and compliance

Previous studies have showed that marine mammals have more compliant lungs (i.e., higher C_L) and a more flexible chest compared with terrestrial mammals (see Table 4.6 in the present study; Fahlman et al., 2017; Olsen et al., 1969; Piscitelli et al., 2013). These anatomical features allow these species to exchange much of their TLC in a single breath, and their VC is close to TLC (Fahlman et al., 2011; Fahlman et al., 2017; Kooyman and Sinnett, 1979; Piscitelli et al., 2010). However, some studies have shown that, even during respiratory efforts following dives or exercise, V_T for most breaths is below VC and only around 20-40% of TLC_{est} (see Table 4.6 in the present study and Figure 4 in Fahlman et al., 2020b). The measured V_T for spontaneous breaths in the present study was lower when compared with the estimated V_T for a number of marine mammals (Figure 4.2), but was between 3% and 43% of TLC_{est}, and reached 50% of TLC_{est} when performing active respiratory manoeuvres. This range for spontaneous V_T is similar to that previously measured in resting marine mammals ranging from 20 to 3600 kg (V_T = 32-43% of TLC_{est}: Fahlman et al., 2017; Kooyman, 1973), and exceeded the 14% of TLC reported for terrestrial mammals (Table 4.6). In addition, the measured V_T for spontaneous breaths was higher than that estimated for terrestrial mammals (Figure 4.2), and correlated with M_b with a mass-exponent close to unity (Table 4.5) as previously reported in otariids (Fahlman and Madigan, 2016; Fahlman et al., 2020b) and cetaceans (Fahlman et al., 2020a). Similarly, the average measured sV_T for spontaneous breaths was higher as compared with that of terrestrial mammals, but was lower than that previously reported (Table 4.6) and estimated from a number of marine mammals (22 ml kg^{-1}: Mortola and Sequin, 2009).

The measured dynamic sC_L from one animal in the present study was higher as compared with previous estimations for land mammals, as previously described in their marine counterparts (see Table 4.6 in the present study, and Table 2 in Fahlman et al., 2017 for more species). While chest compliance was not measured in this study, previous results have shown that the chest in pinnipeds does not significantly contribute to the dynamic values (Fahlman et al., 2014). Thus, obtained results in the present study are consistent with previous studies that suggested an increased ventilatory capacity in

marine mammals with larger V_T and dynamic sC_L as compared with terrestrial mammals, and that most breaths while resting or following active respiratory manoeuvres do not reach TLC.

4.5.4. Respiratory flow and flow-volume relationships

In addition to a flexible thorax and compliant lungs, previous studies on the anatomy and mechanical properties of the respiratory system in marine mammals showed that many species have reinforced conducting airways (Bagnoli et al., 2011; Cozzi et al., 2005; Denison and Kooyman, 1973; Fahlman et al., 2017; Kooyman, 1973; Moore et al., 2014; Piscitelli et al., 2013). These anatomical features would allow for alveolar compression during diving and also adequate gas exchange during high $\dot{V}_{exp}$ and short T_{tot} as compared with terrestrial mammals (Fahlman et al., 2017; Kooyman and Sinnett, 1982; Piscitelli et al., 2010; Stahl, 1967). However, when comparing $\dot{V}_{exp}$ and T_{tot} in marine mammals, there appears to be considerable variability (Fahlman et al., 2017; Ponganis, 2011), possibly as a result of the large diversity in the respiratory anatomical and mechanical adaptations within this group of mammals (Fahlman et al., 2017; Kooyman, 1973; Moore et al., 2014; Piscitelli et al., 2010; Piscitelli et al., 2013). The peak s$\dot{V}$ can be used to compare the ventilatory exchange capacity among different species, and previous studies have showed that some cetaceans and the California sea lion exceed reported s$\dot{V}$ for humans (Table 4.6). While measured $\dot{V}_{exp}$ during active manoeuvres in the present study was higher than that reported for humans (Figure 4.3), the maximal peak s$\dot{V}_{exp}$ was between the reported values for humans, cetaceans and the California sea lion, and those estimated from available data of phocids (Table 4.6). Further studies providing the opportunity to correlate mechanical properties and respiratory function in these species, would help us to understand their respiratory adaptations and the functional consequences on their exchange capacity.

The reported flow-volume relationships for the Pacific walrus in the present study indicated that the flow during active and spontaneous expirations is maintained over most of the V_T, as previously reported for marine mammals (see Figure 4.3 in the present study and results section in Chatper 2 in the present thesis; Fahlman et al., 2019b; Fahlman et al., 2015; Fahlman and Madigan, 2016; Kerem et al., 1975; Kooyman and Cornell, 1981; Kooyman et al., 1975; Kooyman and Sinnett, 1979; Matthews, 1977; Olsen et al., 1969). In contrast, the respiratory mechanics of humans show that the peak expiratory flow during maximal expirations is effort independent, where the peak occurs at high lung

volumes and rapidly drops while lung volume decreases (see Figure 4.3 in the present study; Hyatt et al., 1958; Jordanoglou and Pride, 1968). This flow limitation in humans is related to the increasing flow resistance caused by the compression of the flexible distal airways during emptying of the lungs (Hyatt et al., 1958). Thus, considering the stiffer airways described for marine mammals and the reported flow-volume relationships, it is likely that the flow is not limited by the conducting airways and that the expiration during active and spontaneous respiratory manoeuvres appears to be effort dependent in this group of mammals. However, the results in Chapter II of the present thesis, which aimed to assess respiratory health in bottlenose dolphins (*Tursiops truncatus*) similarly to spirometry methods in humans (Clausen, 1982; Crapo, 1994), showed flow limitations associated with obstructive respiratory disease (see Figure 5.2 in Chapter II). While additional studies should be conducted to determine whether this also translates to other marine mammals, respiratory disease would be expected to have similar consequences in other species, considering the respiratory similarities among this group. Therefore, increased baseline information of normal lung function and flow-volume dynamics in these species would allow evaluation of the mechanical consequences of respiratory disease. This would be beneficial in terms of gaining a better understanding about the respiratory function limitations in marine mammals, and would enhance our effort in the protection of these species through the development of new diagnostic methods.

4.6. Conclusions

The results presented in the current thesis chapter provide additional data about the respiratory capacity in another marine mammal, the walrus, which will add to collective information to improve our understanding of respiratory physiology in marine mammals. While this study reports data on three adult female Pacific walruses, additional information from animals of different age and sex, would be relevant to help improve the understanding of respiratory physiology in this species. The results presented here are in agreement with those reported in California sea lions and suggest a flow limitation when lying on land, and that hydrostatic pressure could help increase $\dot{V}_{exp}$ when resting in water versus on land. In addition, while further studies would help confirm the respiratory function limitations when lying on land as compared with sitting, the results in the present thesis chapter suggest that lung function studies in other semi-aquatic marine mammals should evaluate respiratory function both on land and in water.

4.7. Acknowledgements

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4.8. List of symbols and abbreviations

- C_L = Lung compliance
- f_R = Respiratory frequency
- MAV = Minimum air volume
- M_b = Body mass
- $M_{b\text{-}Exp}$ = Body mass measured during the experimental period
- $M_{b\text{-}LF}$ = Body mass measured the same week as lung function testing
- P_{amb} = Ambient atmospheric pressure
- P_{ao} = Airway opening pressure
- P_{oeso} = Oesophageal pressure
- P_L = Transpulmonary pressure
- sC_L = Specific lung compliance
- STPD = Standard temperature pressure dry conditions
- s$\dot{V}$ = Specific respiratory flow
- s$\dot{V}_E$ = Specific respiratory minute volume
- s$\dot{V}_{exp}$ = Specific expiratory flow
- s$\dot{V}_{insp}$ = Specific inspiratory flow
- sV_T = Mass specific tidal volume
- T_{exp} = Expiratory duration
- T_{insp} = Inspiratory duration
- TLC = Total lung capacity
- TLC_{est} = Estimated total lung capacity
- T_{tot} = Total breath duration
- VC = Vital capacity
- VC_B = Behavioural vital capacity
- $\dot{V}$ = Respiratory flow
- $\dot{V}_E$ = Respiratory minute volume
- $\dot{V}_{exp}$ = Expiratory flow
- $\dot{V}_{insp}$ = Inspiratory flow
- V_T = Tidal volume
- V_{Texp} = Expiratory tidal volume
- V_{Tinsp} = Inspiratory tidal volume

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5.Chapter II: Spirometry as a diagnostic tool in the bottlenose dolphin

Pulmonary function testing as a diagnostic tool to assess respiratory health in bottlenose dolphins (*Tursiops truncatus*)

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Bottlenose dolphin, *Tursiops truncatus* (Montagu, 1821)
Valencia
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5. Chapter II: Spirometry as a diagnostic tool in the bottlenose dolphin

5.1. Abstract

Pulmonary function testing (PFT) was performed in three bottlenose dolphins (*Tursiops truncatus*, one female and two males) under managed care during a 2-year period to assess whether these data provide diagnostic information about respiratory health. Pulmonary radiographs and standard clinical testing were used to evaluate the pulmonary health of each dolphin. The female dolphin (F1) had evidence of chronic pulmonary fibrosis, and one male (M2) developed pneumonia during the study. Pulmonary function data were collected from maximal respiratory efforts in water and from spontaneous breaths while beached. From these data, the flow-volume relationship, the flow measured between 25% and 75% of the expired vital capacity (mid forced expiratory flow, $FEF_{25\%-75\%}$), and the percent of the vital capacity (VC) at the peak expiratory flow ($\%VC_{PEF}$), were evaluated and compared with the diagnostic assessment. For maximal respiratory manoeuvres in water, there were no differences in $FEF_{25\%-75\%}$ or $\%VC_{PEF}$, and the flow-volume relationship showed a consistent pattern for F1. Additionally, $FEF_{25\%-75\%}$ and $\%VC_{PEF}$ decreased by 27% and 52% respectively, and the flow-volume relationship showed clear flow limitations with emerging disease in M2. While spontaneously breathing on land, M2 also showed a 49% decrease in $\%VC_{PEF}$ and changes in the flow-volume relationship, indicating flow limitations following the development of pneumonia. Based on these preliminary results, the present investigation suggests that PFT should be given more attention as a non-invasive and possibly adjunctive diagnostic tool to evaluate lung health of dolphins under managed care and in the wild.

5.2. Introduction

Respiratory disease is a major problem for cetaceans both in the wild and those held under managed care (Baker, 1992; Bogomolni et al., 2010; Howard et al., 1983; Johnson et al., 2009; Sweeney and Ridgway, 1976; Venn-Watson et al., 2012), where the clinical signs are often masked until the animal is severely affected. Methods to diagnose respiratory disease include computed tomography (CT), ultrasound, radiography, bronchoscopy, bronchoalveolar lavage (BAL), tracheal wash, blood and blow samples, and cytology (Ivančić et al., 2014; Johnson et al., 2009; Martony et al., 2017; Reidarson et al., 1998; Smith et al., 2012; Sweeney and Reddy, 2001). While CT may be the gold-standard for diagnostic purposes and has been used in some cases, it is logistically challenging for these species. Portable ultrasound and radiography, on the other hand, are becoming more common, but both have limitations. The former can be done with the animal both in and out of the water, but due to the near-perfect reflection of the sound beam at tissue-air interfaces, only the lung periphery can be evaluated (Martony et al., 2017; Smith et al., 2012). Radiography requires that the animal is on land, which causes lung compression in cetaceans like dolphins, which often weigh 200 kg or more. Additionally, for large animals, high X-ray exposure settings, multiple images, and orthogonal projections are necessary to cover the entire lung field (Van Bonn et al., 2001). Bronchoscopic intervention provides information about potential obstruction and allows the opportunity to obtain biopsies. Blow samples, tracheal washes, or BAL are used to perform cytology or culture the microbiome to assess airway disease. Together with blood samples, these tests provide key information about active disease and the type of infection, e.g., fungal or bacterial, but they do not provide immediate results or information about the functional changes.

Pulmonary function testing (PFT) is commonly used to assess lung health in humans (Crapo, 1994; García-Río et al., 2013; Miller et al., 2005), and has been successfully used in the veterinary field to study pulmonary disease in terrestrial mammals of different sizes (Hoffman, 2002; McKiernan and Johnson, 1992; Rozanski and Hoffman, 1999). This technique may be a useful diagnostic tool in marine species where it is logistically challenging to identify pulmonary disease and assess its progression with CT, radiographs, bronchoscopy, and ultrasound (Fahlman et al., 2019; Gans, 2013). Spirometric PFT is performed by measuring the respiratory flow during one, or several, breathing manoeuvres. In humans, flow and volume limitations are evaluated

by asking the patient to inhale fully to total lung capacity (TLC, l) and then exhale forcefully (García-Río et al., 2013; Miller et al., 2005). A number of pulmonary function indices, and the obtained flow-volume relationships are used to evaluate whether there are deviations from normal patterns.

In the current study, radiographs and clinical tests were used to evaluate respiratory health in three adult bottlenose dolphins. The animals cooperated in PFT in water and provided pulmonary function data for spontaneous and forced breaths, which were then compared with radiographs and clinical tests. The animals included in the study were one female dolphin with evidence of pulmonary fibrosis (F1), one male dolphin that remained healthy throughout the study (M1), and one initially healthy male that contracted pneumonia during the testing period (M2). The two male dolphins also participated in opportunistic PFT while beached and spontaneously breathing.

5.3. Materials and methods

Three adult bottlenose dolphins [*Tursiops truncatus*, (Montagu, 1821)] housed at Siegfried and Roy's Secret Garden and Dolphin Habitat (Las Vegas, NV, USA), participated voluntarily in PFT (spirometry) using a custom-built Fleisch type pneumotachometer designed for dolphins (Mellow Design, Valencia, Spain; See an example in Figure 3.1 in the General methodology section). PFT trials occurred twice per year between April 2017 and April 2019 (six sampling periods). Experimental protocols were approved by the Animal Care and Welfare Committee of Fundación Oceanogràfic de la Comunitat Valenciana (Valencia, Spain, OCE-17-16, and amendment OCE-29-18) and by Siegfried and Roy's Secret Garden and Dolphin Habitat.

5.3.1. Experimental procedures and environmental variables

The three dolphins participated in PFT trials while in water, with the animals either breathing spontaneously, or being asked to make maximal respiratory efforts. PFT trials in water were performed through behavioural cooperation, and no dolphin was restrained during the experimental trial. The two male dolphins also underwent PFT trials while beached for a medical procedure, and opportunistic data from spontaneous breaths were collected. For PFT while beached, the dolphin voluntarily swam into a medical pool and the floor was lifted until the dolphin was out of water. Each trial in water or while beached consisted of one animal remaining stationary, allowing for placement of the pneumotachometer (or spirometer) over the blowhole, and breathing spontaneously or

being asked to make 4-5 maximal respiratory efforts (trained "chuff") while continuous flow measurements were made (see Figure A.1 in the Appendix section for an example). Body mass (M_b, kg) of each animal was measured within the same week as PFT trials. The ambient pressure, and air temperature and humidity (thermometer and hygrometer OH513 Oh Haus & Co.) were measured prior to initiating each experimental trial. The water temperature at the facility housing the animals was measured through the experimental period by the staff at Siegfried and Roy's Secret Garden and Dolphin Habitat, and the average (± standard error, s.e.m.) was 24.7 ± 2ºC (range = 24-26ºC).

5.3.2. General health monitoring and routine clinical pathology

To assess the health of each dolphin at the time of each PFT trial, specific clinical samples were collected, analysed, and interpreted. Complete blood cell counts, serum chemistries (analysed at IDEXX Laboratories, Inc., NV, USA), sedimentation rates, and sputum cytology (analysed onsite by Siegfried and Roy's Secret Garden and Dolphin Habitat laboratory technician staff) were performed within ± 14 days of the PFT exams.

5.3.3. Radiography

For thoracic radiographs acquisition, the three dolphins each voluntarily swam into a submerged stretcher, were manually lifted from the water, and placed in sternal and lateral recumbency during image acquisition. For each dolphin, numerous (11-20) radiographs were obtained using a portable X-ray unit (MinXRay TR90B portable X-ray unit, MinXRay, Inc., IL, USA; Canon CXDI-70C wireless digital radiography detector, Canon USA, Inc., CA, USA; VetRocket X1 Portable DR system, VetRocket, LLC, CA, USA) as necessary to permit assessment of all lung fields. Orthogonal views were acquired for all animals and included dorsoventral and lateral projections obtained through vertical and horizontal beam techniques. No complications were observed following prompt return of each animal into the water. Following image acquisition, results were evaluated in an optimized environment using a commercially available, FDA-approved DICOM viewer (MacPro with Apple Thunderbolt Display, Apple, CA, USA; Osirix MD v.8.0.1, Pixmeo, Switzerland) by a board-certified veterinary radiologist (S. Dennison) with extensive experience in marine mammal diagnostic imaging. Radiographs were evaluated to determine whether the thorax, and specifically the pulmonary parenchyma and airways, were normal or abnormal. If abnormal, further characterization of the pattern present was classified as unstructured interstitial (a poorly organized change in the parenchyma),

structured interstitial (nodular change) or alveolar (consolidation of a region or regions), patterns per veterinary radiographic interpretation convention.

5.3.4. Spirometry tests and respiratory variables

The procedures and components for measuring respiratory flow ($\dot{V}$, l s^{-1}), total breath duration (T_{tot}, ms), and to estimate tidal volume (V_T, l) converted into standard temperature and pressure dry (STPD, Quanjer et al., 1993), are summarized in the General methodology section of the present thesis and detailed in Chapter I. To assess changes in respiratory function during disease, the flow-volume relationship was evaluated, and two functional indices were defined. One of these was the flow measured between 25% and 75% of the expired vital capacity (VC, l) or the mid forced expiratory flow ($FEF_{25\%-75\%}$, l s^{-1}) (García-Río et al., 2013; Miller et al., 2005). The second index was obtained from a variable used in human spirometry, the peak expiratory flow (PEF, l s^{-1}) (García-Río et al., 2013; Miller et al., 2005), but adjusted for the respiratory mechanics and capacity in dolphins (Fahlman et al., 2017): the percent of the expired VC at the PEF ($\%VC_{PEF}$).

5.3.5. Data assessment and statistical analysis

Only respiratory manoeuvres composed by an expiration and inspiration were included in the analysis. For experiments in water, the three maximal expiratory efforts of each trial were used to evaluate the flow-volume relationship and calculate $FEF_{25\%-75\%}$ and $\%VC_{PEF}$. For beached trials, the flow-volume relationship of all voluntary breaths was obtained, and the three most similar manoeuvres were included for the analysis. Temporal changes in PFT variables (expiratory and inspiratory duration [T_{exp} and T_{insp}, respectively], expiratory [$\dot{V}_{exp}$] and inspiratory [$\dot{V}_{insp}$] $\dot{V}$ and V_T [V_{Texp} and V_{Tinsp}, respectively]) and PFT indices ($FEF_{25\%-75\%}$ and $\%VC_{PEF}$) were assessed using linear mixed-effects models (lme, R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, v.3.6.1, 2019). Trial date and respiratory effort (voluntary or maximal/chuff) were considered as independent fixed covariates, and the individual animal was treated as a random effect to account for the correlation between repeated measurements on the same individual (Littell et al., 1998). A univariate analysis on each independent variable was initially performed, and only those variables with a *p*-value < 0.10 were considered in a multivariate analysis (Wald's test). Best models for a multivariate analysis were selected based on the log-likelihood ratio test and the Akaike information criterion, and significant parameters assessed by the t-value between the

estimate and its standard error. In this study p-values ≤ 0.05 were considered as significant and all values are reported as means ± standard deviation (s.d.) unless otherwise stated.

5.4. Results

For the three participating dolphins, sex, morphometrics, and year of birth are reported in Table 5.1. The dolphins participated in a total of 22 PFT trials during the experimental period. Mean (± s.e.m.) air temperature, environmental pressure and humidity during trials (n = 22) were, respectively, 22.0 ± 1.6 °C (range = 12–20°C), 101.4 ± 0.2 kPa (100.2-102.6 kPa), and 30.0 ± 4.0% (9-42%).

Table 5.1. Sex, morphometrics and year of birth of bottlenose dolphins.

Animal ID	Sex	M_b (kg)	SL (cm)	YOB (year)
F1	F	231 ± 8	262	1975*
M1	M	186 ± 8	240	2011
M2	M	177 ± 4	242	2011

For the three adult bottlenose dolphins (*Tursiops truncatus*) participating in pulmonary function testing, animal identification (Animal ID), sex (M/F), average (± s.d.) body mass (M_b) measured the same week of each trial, straight length (rostrum to fluke notch, SL), and year of birth (YOB). *The birth year of the female was unknown but estimated.

5.4.1. Clinical cases summary: routine clinical pathology and radiography

The female dolphin (F1) was previously diagnosed with chronic bronchitis and bouts of pneumonia through radiographs and routine clinical pathology (increased white cell count and sedimentation rate, and culture positive for *Aspergillus* spp.). During the experimental period, this dolphin presented with a chronic decreased serum iron. Additionally, unstructured interstitial abnormalities were identified on radiographs (Table 5.2), with the most applicable differentials being fibrosis, infection, or low grade, chronic airway/parenchymal inflammation. However, as the degree of change did not vary significantly in the following radiography evaluations, fibrosis was most likely, based on radiographs. Treatment administered previously (several years prior to study dates) and during the study period for a urinary tract infection and pulmonary disease included posaconazole (dosed at ~3 mg kg^{-1}) and Clavamox (dosed at ~6 mg kg^{-1} twice a day).

The healthy male bottlenose dolphin (M1) had previously been diagnosed with suspected emerging pneumonia through thoracic radiographs (spring 2016), that showed resolution of disease in July 2016. The white blood cell count, serum iron, sedimentation rate, fibrinogen and blow cytology were within normal range during the study period. All

radiographs performed were interpreted as normal, except for an emerging abnormal pattern interpreted as possible pneumonia in January 2019, which had radiographically resolved in March 2019 (Table 5.2). The dolphin was assumed symptom free but received voriconazole (dosed at ~2 mg kg^{-1} once a day) from January until March following the radiographic findings.

Male dolphin (M2) had previously been diagnosed with intermittent bronchitis and bouts of pneumonia that showed resolution of disease on radiographs in June 2016. During the experimental period, the erythrocyte sedimentation rate was within normal range, but was markedly increased (60 mm $hour^{-1}$, normal range = 4-17 mm $hour^{-1}$) in April 2018. Radiographs showed that both lung fields were normal throughout 2017, but unstructured and structured abnormalities, suggestive of pneumonia, were identified in both lungs in January 2018 (Table 5.2). Radiographs showed persistent pulmonary pathology with improvement on repeated studies in May and June, that radiographically worsened in August 2018, and improved at the end of 2018 and the beginning of 2019. The dolphin was treated for pneumonia from January 2018, beginning with voriconazole (dosed at ~2 mg kg^{-1} once daily), which was switched to posaconazole (dosed at ~4 mg kg^{-1} once daily) after 5 months when the animal showed persistent pulmonary pathology on chest radiographs.

Table 5.2. Summary of radiographic pulmonary assessment in bottlenose dolphins.

Date of radiographs	Participating dolphin		
	F1	M1	M2
July-August 2016	UI	N	N
February-March 2017	SI[a]	NA	N[a]
August 2017	NA	NA	N
October 2017	NA	N[a]	NA
January 2018	NA	N	UI and SI
May-June 2018	SI[b]	NA	UI and SI[a]
Jul 2018	UI	NA	NA
August 2018	NA	NA	UI and SI[a]
November 2018	NA	NA	UI and SI
January 2019	NA	UI	UI and SI[a]
March 2019	NA	NA	NA

Radiographic results obtained from thoracic examinations in three adult bottlenose dolphins (*T. truncatus*) desensitized and trained to perform the procedures. NA: not assessed; N: normal; SI: structured interstitial; UI = unstructured interstitial. [a]Radiographs taken ± 1-30 days either before or after the PFT. [b]Radiography taken after 56 days of the PFT. See Table 5.1 for details of individual dolphins.

5.4.2. Spirometry in water

The three bottlenose dolphins participated in 18 trials while in water, and a total of 122 spontaneous breaths were collected and included in the analysis (Table 5.3). For spontaneous breaths, there were no differences between T_{insp} (359 ± 67 ms) and T_{exp} (498 ± 96 ms), $\dot{V}_{insp}$ (13.6 ± 3.3 l s^{-1}) and $\dot{V}_{exp}$ (20.1 ± 4.0 l s^{-1}), or V_{Tinsp} (5.3 ± 1.8 l) and V_{Texp} (4.8 ± 1.8 l) between dolphins with or without pulmonary disease ($p > 0.1$ for all). A total of 162 maximal respiratory efforts were collected during trials in water, and the three maximal breathing efforts for each trial (n = 18 for each dolphin, Table 5.4) were included in the analysis. For maximal breathing efforts, there were no systematic changes over the experimental period for T_{tot}, $\dot{V}$, or V_T for any dolphin ($p > 0.1$ for all). However, the average mass-specific V_T (sV_T, ml kg^{-1}) and $\dot{V}$ ($s\dot{V}$, ml s^{-1} kg^{-1}) for both, inspiratory (sV_{Tinsp} and $s\dot{V}_{insp}$, respectively) and expiratory (sV_{Texp} and $s\dot{V}_{exp}$, respectively) phases, were significantly lower (least squares regression followed by Bonferroni corrected post hoc test, $df = 2$) in F1 (sV_{Tinsp} = 23 ± 3 ml kg^{-1}; sV_{Texp} = 24 ± 3 ml kg^{-1}; $s\dot{V}_{insp}$ = 61 ± 10 ml s^{-1} kg^{-1}; $s\dot{V}_{exp}$ = 103 ± 12 ml s^{-1} kg^{-1}, $p < 0.01$ for all), as compared with the two males (sV_{Tinsp} = 56-57 ml kg^{-1}; sV_{Texp} = 56-60 ml kg^{-1}; $s\dot{V}_{insp}$ = 103-112 ml s^{-1} kg^{-1}; $s\dot{V}_{exp}$ = 250-305 ml s^{-1} kg^{-1}).

For M1 or F1 neither $FEF_{25\%-75\%}$ nor $\%VC_{PEF}$ changed systematically with repeated trials ($p > 0.05$ for all tests, Figure 5.1). For M2, on the other hand, both $FEF_{25\%-75\%}$ ($\chi^2 = 10.2$, $df = 1$, $p < 0.01$) and $\%VC_{PEF}$ ($\chi^2 = 18.6$, $df = 1$, $p < 0.001$) decreased by 27% and 52%, respectively, for dates when this dolphin was diagnosed with pneumonia (Figure 5.1). For the dolphins with pulmonary disease (M2 and F1), $FEF_{25\%-75\%}$ ($\chi^2 = 20.5$, $df = 1$, $p < 0.001$) and $\%VC_{PEF}$ ($\chi^2 = 22.8$, $df = 1$, $p < 0.001$) were 36% and 61% lower, respectively, compared with the healthy animal.

The flow-volume relationship was visually evaluated for the three maximal respiratory efforts of each trial in water. In the dolphin with pulmonary fibrosis (F1), the flow-volume relationship showed a consistent shape, reduction in VC, and flow abnormalities during all testing periods (Figure 5.2A). In the healthy male dolphin (M1) the shape was consistent between trials and indicated no flow or volume limitations (Figure 5.2B). A normal flow-volume relationship was seen in M2 during the trials in 2017, when the dolphin was healthy, but flow limitations appeared during the testing in April 2018, followed by a gradual recovery towards a normal shape during 2019 (Figure 5.2C).

Table 5.3. Lung function for spontaneous breaths in bottlenose dolphins in water.

Animal ID	Nº	Breath duration (ms)		$\dot{V}$ (l s^{-1})		V_T (l)	
		Exp	Insp	Exp	Insp	Exp	Insp
F1	39	361 ± 51	491 ± 59	16.9 ± 3.1	11.5 ± 2.1	4.0 ± 1.0	4.4 ± 0.8
M1	38	369 ± 79	529 ± 144	21.1 ± 4.4	14.2 ± 2.5	5.2 ± 1.9	5.7 ± 2.2
M2	45	349 ± 79	476 ± 71	22.3 ± 2.2	15.1 ± 4.3	5.3 ± 2.1	5.7 ± 2.1

For the three adult bottlenose dolphins (*T. truncatus*) participating in pulmonary function testing while in water, animal ID, total number of collected breaths (Nº) and average (± s.d., n = 6 trials for each dolphin) of expiratory (Exp) and inspiratory (Insp) breath duration, respiratory flow ($\dot{V}$), and tidal volume (V_T) for spontaneous breaths in water.

Table 5.4. Lung function for maximal respiratory efforts in bottlenose dolphins in water.

Animal ID	Breath duration (ms)		$\dot{V}$ (l s^{-1})		V_T (l)	
	Exp	Insp	Exp	Insp	Exp	Insp
F1	411 ± 41	514 ± 50	23.8 ± 2.8	14.0 ± 2.2	5.6 ± 0.6	5.4 ± 0.7
M1	394 ± 54	697 ± 106	46.5 ± 9	19.2 ± 3.3	10.4 ± 1.9	10.5 ± 1.4
M2	420 ± 104	704 ± 66	54.0 ± 8.3	19.8 ± 2.7	10.7 ± 1.8	10.1 ± 1.4

For the three adult bottlenose dolphins (*T. truncatus*) participating in pulmonary function testing while in water, animal ID and average (± s.d., n = 6 trials for each dolphin) of expiratory (Exp) and inspiratory (Insp) breath duration, respiratory flow ($\dot{V}$), and tidal volume (V_T) for maximal respiratory efforts (chuffs) in water.

5.4.3. Spirometry on land (beached)

In October 2017 and April 2018, two animals (M1 and M2) were beached for a physical examination and PFT was opportunistically performed during spontaneous breathing (n = 4 trials in total). Three breaths per trial were used to evaluate $FEF_{25\%-75\%}$, $\%VC_{PEF}$, and the flow-volume relationship. For M1, there were no significant changes in $FEF_{25\%-75\%}$ or $\%VC_{PEF}$ (Figure 5.3), and the shape of the flow-volume relationship was consistent during spontaneous breaths while beached and in water (Figure 5.4). When M2 beached, the $\%VC_{PEF}$ (χ^2 = 13.1, df = 1, p < 0.001) significantly decreased by 49% in April 2018, at which time this animal was diagnosed with pneumonia (Figure 5.3). For the same dolphin, the flow-volume relationship appeared normal while in water, but abnormalities were apparent during spontaneous breaths while beached in April 2018 (Figure 5.4).

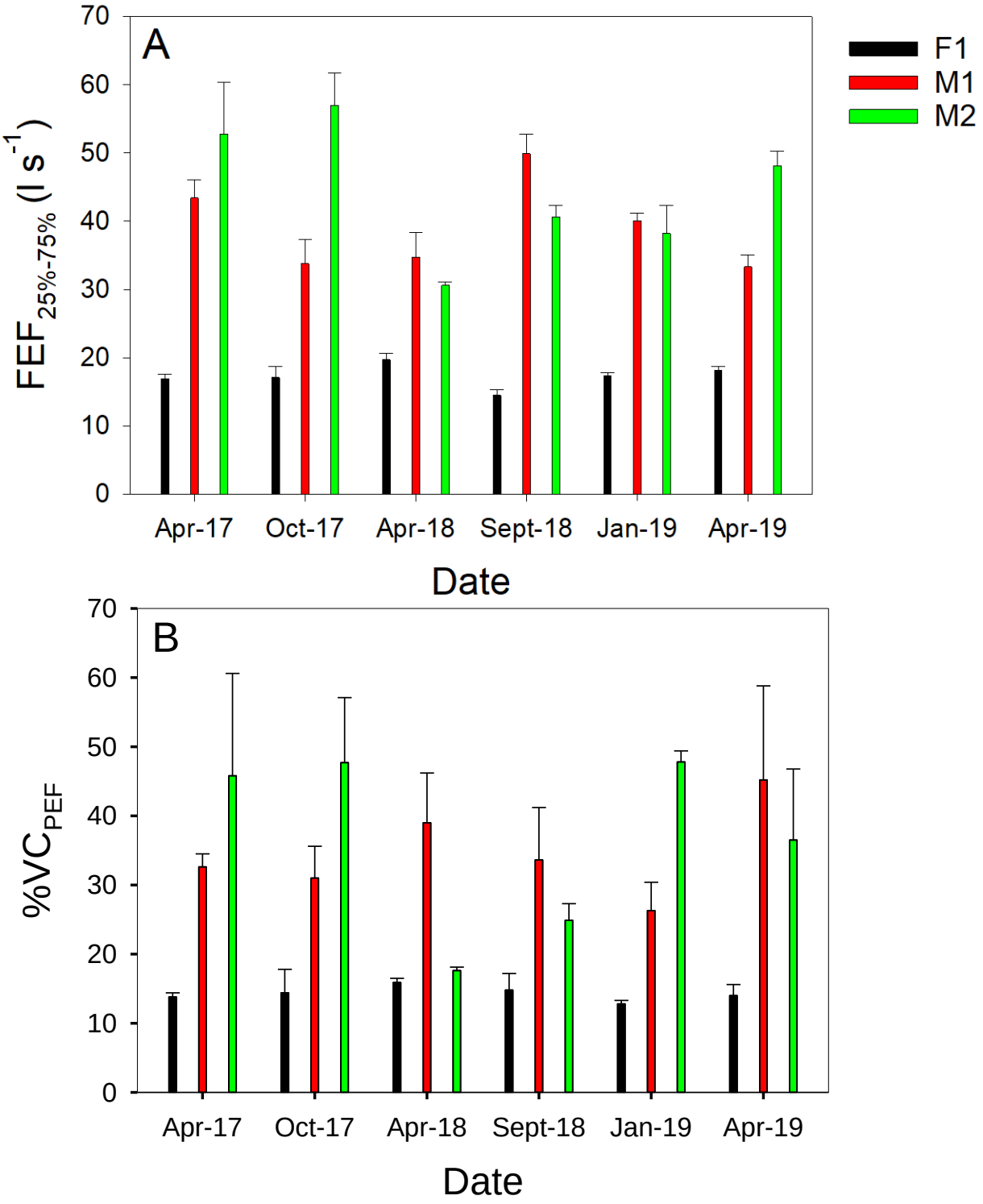

Figure 5.1. Pulmonary function testing indices for maximal respiratory efforts from bottlenose dolphins in water.
Average (± s.d., n = 18 maximal respiratory efforts per each dolphin) of A) mid forced expiratory flow ($FEF_{25\%-75\%}$) and B) percent of expired vital capacity at the peak expiratory flow ($\%VC_{PEF}$) obtained from the three maximal respiratory efforts performed in water between April 2017 and April 2019 from three adult bottlenose dolphins (*Tursiops truncatus*): a dolphin diagnosed with pulmonary fibrosis (F1), a healthy dolphin (M1), and a dolphin diagnosed with pneumonia in January 2018 and with active disease in April 2018 (M2).

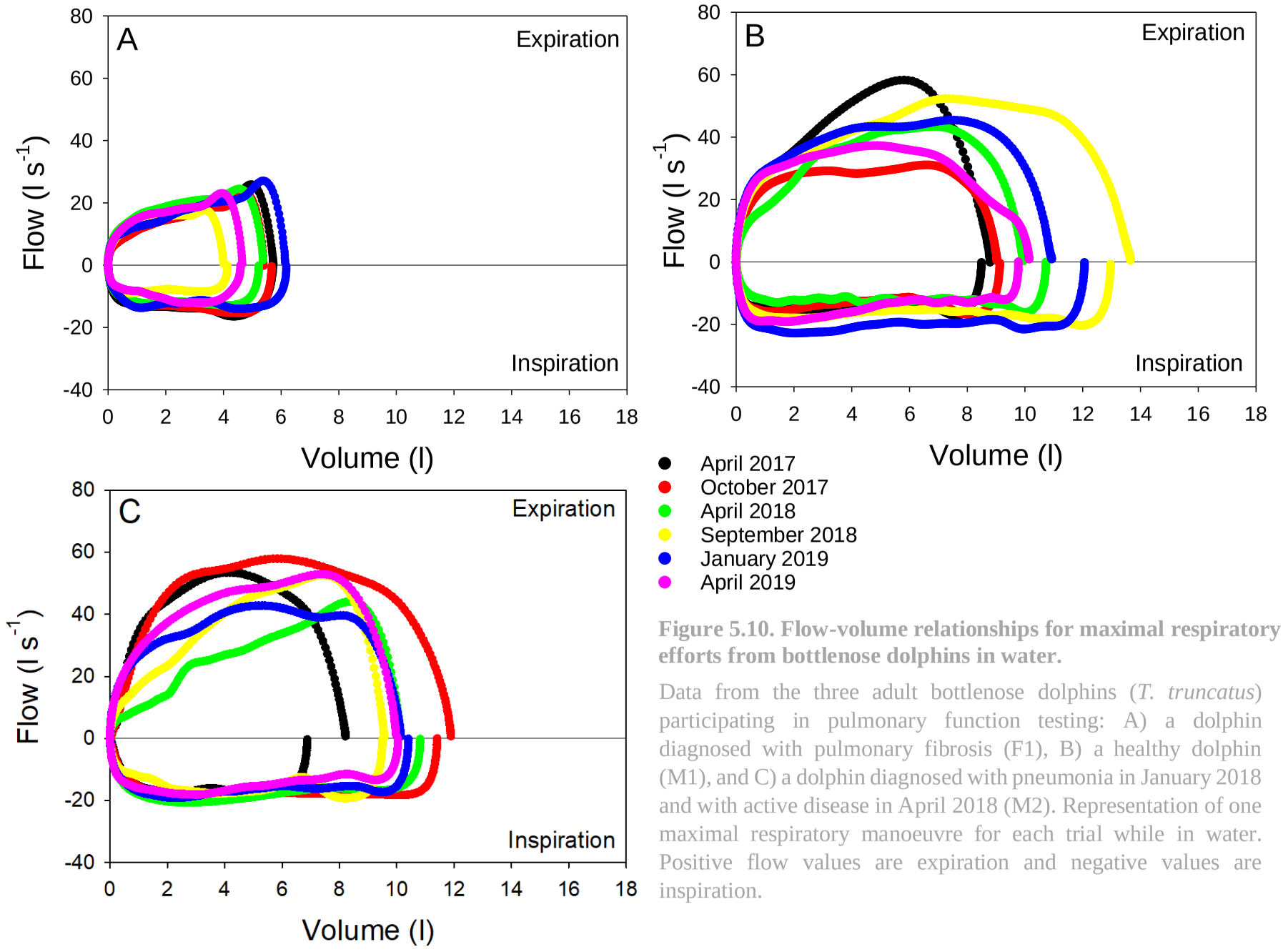

Figure 5.10. Flow-volume relationships for maximal respiratory efforts from bottlenose dolphins in water.

Data from the three adult bottlenose dolphins (*T. truncatus*) participating in pulmonary function testing: A) a dolphin diagnosed with pulmonary fibrosis (F1), B) a healthy dolphin (M1), and C) a dolphin diagnosed with pneumonia in January 2018 and with active disease in April 2018 (M2). Representation of one maximal respiratory manoeuvre for each trial while in water. Positive flow values are expiration and negative values are inspiration.

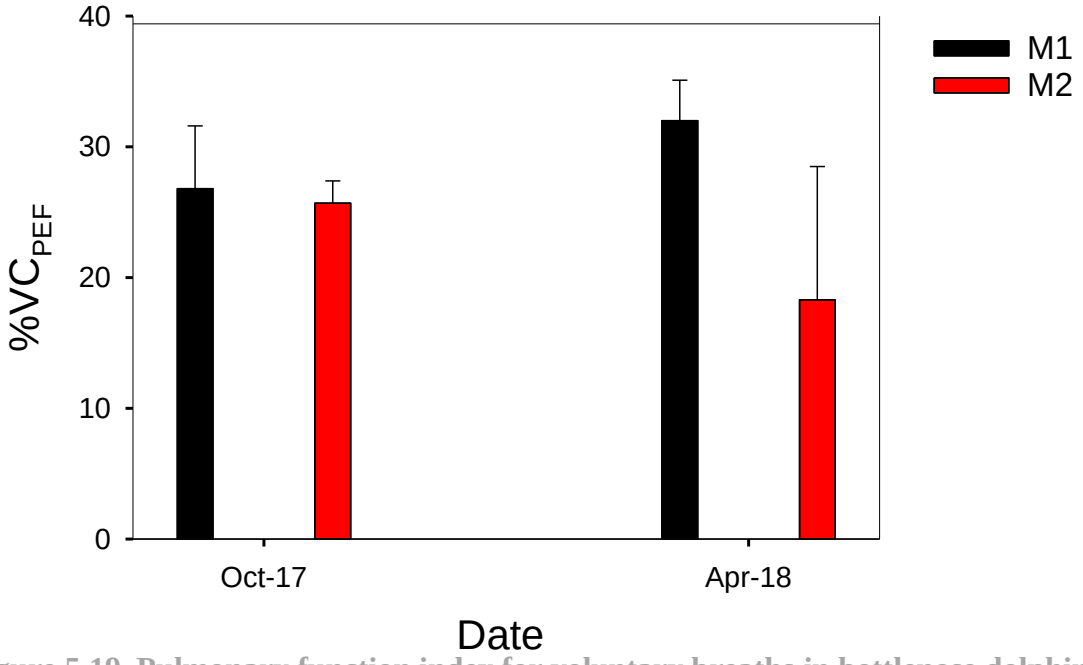

Figure 5.19. Pulmonary function index for voluntary breaths in bottlenose dolphins while beached.
Average (± s.d., n = 2 trials per each dolphin) of the expired vital capacity at the peak expiratory flow ($\%VC_{PEF}$) obtained from three voluntary breaths while beached (October 2017 and April 2018) from two adult bottlenose dolphins (*T. truncatus*): a healthy dolphin (M1), and a dolphin diagnosed with pneumonia in January 2018 and with active disease in April 2018 (M2).

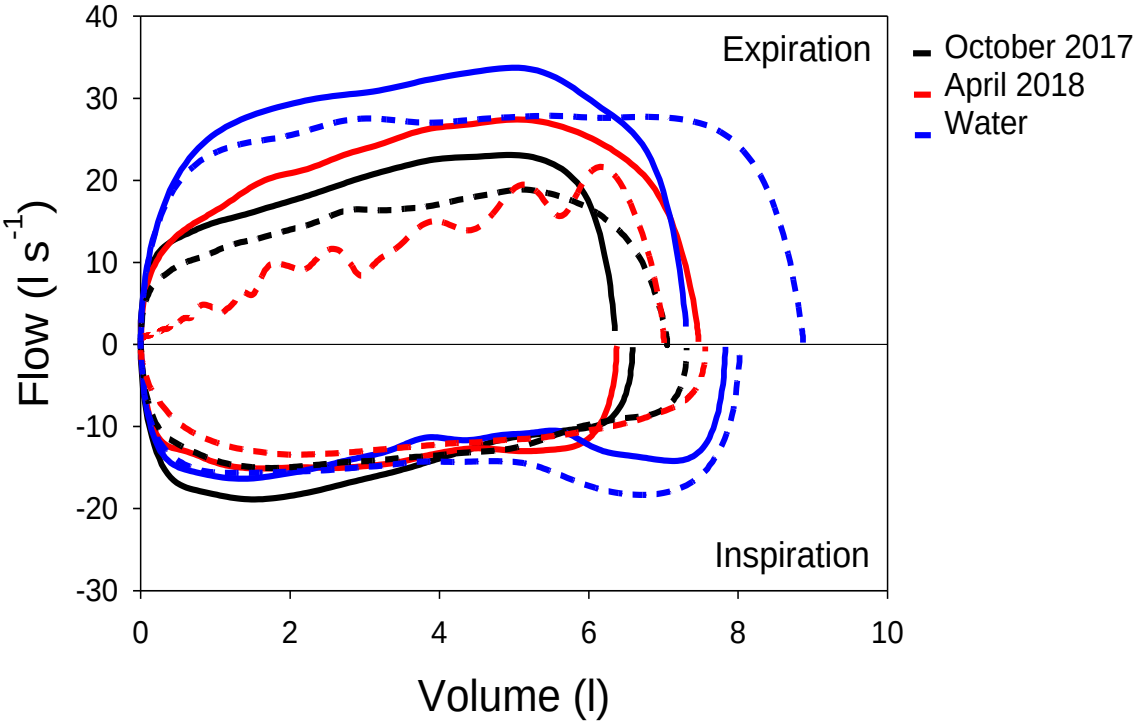

Figure 5.28. Flow-volume relationships for voluntary breaths from bottlenose dolphins.
Voluntary breaths while beached (black and red lines) and in water (blue lines) from two adult bottlenose dolphins (*T. truncatus*): a) a healthy dolphin (M1, solid line) and b) a dolphin diagnosed with pneumonia in January 2018 and with active disease in April 2018 (M2, dashed line). Representation of one maximal respiratory manoeuvre for each trial. Positive flow values are expiration and negative values are inspiration.

5.5. Discussion

PFT is a simple and non-invasive method to study respiratory physiology in voluntarily participating cetaceans (Fahlman et al., 2019; Fahlman et al., 2015; Fahlman et al., 2017; Kooyman and Cornell, 1981; Olsen et al., 1969). These methods are also commonly used in humans to diagnose a variety of pulmonary diseases (Crapo et al., 1981; Miller et al., 2005), and have been adapted for use in some terrestrial mammals (e.g., cat, dog, and horse: Balakrishnan and King, 2014; Herholz et al., 2002; Hoffman, 2002; Hoffman and Mazan, 1999; McKiernan and Johnson, 1992; Willoughby and McDonell, 1979). For these reasons, PFT has been considered a potentially useful adjunctive diagnostic tool in veterinary medicine to assess lung health in marine mammals (Gans, 2013; Van Elk et al., 2001). In this pilot study PFT was evaluated in 3 bottlenose dolphins as an adjunctive diagnostic tool to assess respiratory health. The PFT was compared with standard veterinary methods (e.g., thoracic radiographs, blood count, and blow cytology and culture) to evaluate pulmonary health. These preliminary results suggest that PFT could have the potential to aid in diagnosis, evaluate functional changes, and assess treatment efficacy in managed care dolphins trained to exhale maximally. In addition, further research is necessary to verify if this tool may also provide adjunctive diagnostic information in stranded wild dolphins while breathing spontaneously.

5.5.1. General pulmonary health evaluation: benefits and limitations

Pulmonary parenchymal disease (usually pneumonia) in cetaceans is predominantly inflammatory in nature, with underlying infection identified as the likely initiating cause in many cases (McBain, 2001; Sweeney and Reddy, 2001; Venn-Watson et al., 2012). As such, indicators of inflammation are generally detected in routine haematology, serum chemistry analysis, and blow samples from forceful expiration (Dierauf and Gulland, 2001; Sweeney and Reddy, 2001). Digital radiography is a modality readily available and frequently used to assess dolphins for evidence of pulmonary disease in many marine mammal facilities in the USA. While CT is considered the medical gold standard for radiological evaluation of the lung parenchyma (Ivančić et al., 2014), assessment using CT was not logistically feasible. The female dolphin (F1) showed no signs of active inflammatory disease on blow cytology, but showed a decreased serum iron and abnormal radiograph findings interpreted as pulmonary fibrosis throughout the study. For M1, no abnormal clinical values were documented with haematology, clinical chemistry or blow cytology throughout the study. However, in

January 2019, an emerging abnormal pattern was detected through radiography, which was interpreted as a possible low degree of pneumonia that resolved by March 2019. Clinical values for M2 were within the normal range, except the erythrocyte sedimentation rate, which was significantly elevated when the animal was diagnosed with pneumonia through radiography. Possible methods to classify the type of pneumonia include fungal immunodiffusion assay, BAL, or bronchoscopy (Reidarson et al., 1998), but none of these clinical tests were performed. This highlights the limitation of routine diagnostic methods, which appear not to be sensitive in all cases. Thus, complementary techniques to evaluate functional changes in pulmonary health should be considered.

5.5.2. PFT variables and indices for clinical assessment

While standard PFT does not provide aetiologic information about the disease, it quantifies the degree of severity and the progression of the respiratory system disorder, and, in some cases, is able to define where in the respiratory airways the disease is located (Balakrishnan and King, 2014). For example, in human medicine, maximal inspiratory efforts are used to assess respiratory disease of the upper airways, while expiratory efforts help identify dysfunction of the lower airways. In the present study, operant conditioning only assured data collection from maximal expiratory efforts. Therefore, these maximal expiratory manoeuvres were evaluated based on methods developed in human medicine (García-Río et al., 2013; Miller et al., 2005), but modified for the respiratory function in dolphins, to assess flow and volume limitations associated with disease in the lower airways of the respiratory system. In human medicine, volume-related indices, e.g., the maximal volume of exhaled air (forced vital capacity, FVC), and the forced expiratory volume in the first second (FEV_1), are important to assess pulmonary health in the lower airways, where severe obstructive or restrictive respiratory disease results in a decrease in both indices (García-Río et al., 2013). However, cetaceans have high expiratory flows at all lung volumes (Figure 5.2B), a VC that is close to TLC, and breath durations of less than one second (Fahlman et al., 2019; Fahlman et al., 2015; Fahlman et al., 2017; Kooyman and Cornell, 1981; Kooyman and Sinnett, 1979; Olsen et al., 1969). Consequently, the traits of cetacean respiratory system make it difficult to assess lung health through FEV_1. On the other hand, while V_T during forced breaths can be used as an estimate of FVC, no differences were detected in V_T throughout the experimental period in any of the dolphins. However, the sV_T during forced breaths in F1 was considerably lower than in M1 and M2. This could be a consequence of the reduction in

VC due to pulmonary fibrosis (restrictive disease), and/or be related to differences in the respiratory functionality due to sex or age, as commonly found in humans (Ruivo et al., 2009; Sharma and Goodwin, 2006). Whether this is a sign of pulmonary disease, sex, or age cannot be determined with this limited data set, baseline values of healthy dolphins over a range of ages would be needed for comparison.

Other standard flow-related indices used in human medicine to assess pulmonary health in the lower airways include $FEF_{25\%-75\%}$ and PEF, where obstructive or restrictive disease results in a decrease in both indices (García-Río et al., 2013). $FEF_{25\%-75\%}$ provides information about flow values in the mid part of the forced expiration. Furthermore, $FEF_{25\%-75\%}$ details the mechanical function of the lungs through evaluation of the flow-volume relationship across different volumes. Therefore, in the present study it was hypothesized that this index would provide valuable information about lung health in the dolphins. The PEF index, on the other hand, only gives information about the maximal $\dot{V}_{exp}$ during a forced manoeuvre. Assuming that the $\dot{V}_{exp}$ during forced breaths provides an estimate of PEF, there were no systematic changes in this PFT index for any of the dolphins during the experimental period, but lower $s\dot{V}_{exp}$ values were seen in F1 compared with M1 and M2, which could be also related with the disease, sex, or age. In addition, the PEF can be reached at both high and medium volumes in healthy dolphins (Figure 5.2B). However, in dolphins with respiratory disease, on the other hand, the PEF only occurred at high volumes during the forced expiratory manoeuvre, with the flow then rapidly decreasing with reduced lung volume (Figures 2A and 2C). Therefore, the $\%VC_{PEF}$ was used as another index for pulmonary disease detection, where a lower percentage of expired volume in the PEF is expected in animals with lung disease.

5.5.3. Evaluation of respiratory health: maximal respiratory manoeuvres while in water

The preliminary data presented in this thesis chapter suggest that PFT could be a useful method to assess respiratory health through evaluation of the functional capacity from forced breaths in dolphins under managed care (Figures 5.1 and 5.2). For the female diagnosed with pulmonary fibrosis (F1), neither index changed temporally, but both indices were lower as compared with the values in the healthy dolphins (Figure 5.1), similar to the results for sV_T and $s\dot{V}_{exp}$. The consistent reduction in $FEF_{25\%-75\%}$, $\%VC_{PEF}$, and forced V_T and $\dot{V}$ suggest a suitable correspondence with the radiographical findings, as chronic fibrosis is a pulmonary restrictive disease that reduces VC. Additionally, the shape of the flow-volume relationship for F1 also suggested obstructive disease, where

the PEF occurred at high lung volume and the $\dot{V}_{exp}$ rapidly decreased with lung volume (Figure 5.2A).

For M1, none of the indices changed throughout the study, even during the time (January 2019) when the radiographs indicated unstructured interstitial changes interpreted as low degree of pneumonia. The flow-volume relationship for M1 indicated a healthy lung that could maintain high flow over the entire lung volume (Fahlman et al., 2017) and no evidence of pulmonary disease (Figure 5.2B). Additionally, no changes in the clinical routine health assessment were found, and the pneumonia quickly resolved. Therefore, these results could suggest that the emerging pneumonia was of low severity and did not alter lung function to be detectable through PFT.

For M2, both $FEF_{25\%-75\%}$ and $\%VC_{PEF}$ decreased substantially from 2017 until April 2018, at which time the dolphin had been diagnosed with pneumonia. Both $FEF_{25\%-75\%}$ and $\%VC_{PEF}$ then increased in September 2018 and January 2019, suggestive of recovery (Figure 5.1), which was confirmed by radiography assessment. The flow-volume relationship for M2 indicated an obstructive pattern in April 2018, and progressive recovery during 2019 (Figure 5.2C). However, the pneumonia (restrictive disease) did not cause a reduced VC for M2, and parenchymal inflammation (e.g., pneumonia) is commonly related to concomitant obstructive diseases in the respiratory airways. Thus, while both F1 and M2 were diagnosed with restrictive disease through routine diagnostic methods, the lung function testing also suggested lower airway obstruction in both dolphins. Therefore, PFT added diagnostic information about the type and location of the disease.

5.5.4. Evaluation of respiratory health: spontaneous breaths while beached

In human medicine, respiratory disease is evaluated by spirometry through maximal respiratory efforts. In small terrestrial mammals, or in human paediatric medicine, methods have been developed to identify airway obstruction during spontaneous breathing, where reliable cooperation of the patient to perform maximal respiratory efforts is difficult (Abramson et al., 1982, Balakrishnan & King 2014). The tidal breathing flow-volume loop (TBFVL) test has been developed to evaluate flow obstruction in human infants (Abramson et al., 1982), and has also been tested in small domestic animals (McKiernan & Johnson 1992, Balakrishnan & King 2014). It has been suggested that the sensitivity of the TBFVL test could be enhanced by increasing the external pressure on the chest wall in patients that are not able to perform maximal

respiratory efforts (Abramson et al., 1982). While dolphins are beached, the external pressure on the chest is increased due to their own weight. For M1 and M2 when healthy and spontaneously breathing, there was a decrease in VC and a steeper slope of the flow-volume relationship while beached as compared to when in water (Figure 5.4). Thus, the extra pressure on the chest could lead to an increase in the respiratory effort, which resulted in the observed changes for both dolphins while healthy. Between beached testing dates, there were no changes in any of the PFT indices and the flow-volume relationship for M1. On the other hand, there was a significant decrease in the $\%VC_{PEF}$ for M2 (Figure 5.3), and changes in the slope of the flow-volume relationship consistent with flow abnormalities for the trial date when the animal had been diagnosed with pneumonia (Figure 5.4). These results suggest that the combination of respiratory effort and pulmonary disease increased the respiratory limitation in M2, resulting in the observed results. In addition, these pilot data suggest that the $\%VC_{PEF}$ index could also be sensitive during spontaneous breaths with increased respiratory effort while beached. For example, when M2 contracted pneumonia, the PEF reached its maximum at the beginning of the expiration, similarly to forced breaths in water. However, the $FEF_{25\%-75\%}$ is related to the slope of the flow-volume relationship during the expiration. Therefore, the steeper slope of the flow-volume relationship due to the additional pressure on the chest while beached could make it difficult to evaluate lung disease through $FEF_{25\%-75\%}$ while spontaneously breathing on land, as the slopes when M2 was healthy and when diagnosed with pneumonia were similar (Figure 5.4). Consequently, extensive data from other clinical cases are necessary for a deeper understanding about the relation between $\%VC_{PEF}$, $FEF_{25\%-75\%}$ and lung disease while spontaneously breathing on land.

PFT has been suggested as a non-invasive method to assess lung health in marine mammal veterinary medicine. The methodology is still being developed, requires post-processing, and offers some challenges when applied to trained animals. For example, placement of the mask over the blow hole (or nostrils in pinnipeds) may cause a certain amount of discomfort. Additionally, the training itself may cause a bias through conditioning the respiratory manoeuvres, which is difficult to evaluate. Although desensitization helps improve comfort and repeatability between testing dates in the pulmonary function values, the same is not true for wild individuals. Some researchers consider results from animals under human care to have limited value. However, certain information is nearly impossible to collect from wild populations, where it is logistically

and ethically challenging to perform controlled studies. Studying physiology and function in animals under managed care includes access to complete medical histories, as well as the ability to perform more comprehensive diagnostics than is possible with wild animals. The animals can be trained to participate voluntarily for baseline data collection with minimal stress and to cooperate to perform certain manoeuvres e.g., maximal respiratory efforts made in cetaceans or pinnipeds (see Chapter I in the present thesis; Fahlman et al., 2019; Fahlman et al., 2015; Fahlman and Madigan, 2016; Fahlman et al., 2020; Kerem et al., 1975; Kooyman and Cornell, 1981). Such cooperation is possible with trained animals, but not in wild populations (Fahlman et al., 2018a; Fahlman et al., 2018b).

5.6. Conclusions

The preliminary results presented in this thesis chapter indicate that spirometry has the potential to improve and provide additional information about the type (obstructive or restrictive) and location (lower airways) of the pulmonary disease in dolphins. While PFT development would require training to generate maximal respiratory efforts for dolphins in water, these results additionally suggest that this non-invasive method should be further studied to define or characterize respiratory health in stranded cetaceans while breathing spontaneously. These results highlight the importance of studying trained animals under managed care, where development and validation of new equipment can be performed under controlled situations before being applied to wild animals. Therefore, further research with the knowledge gained in the present study is necessary to validate the technique: characterization of lung function indices, classification of the flow-volume relationship related to obstructive and restrictive pulmonary diseases, and determination of reference values for healthy animals of different sex and age (young, mature, and elderly). In conclusion, the development of PFT in marine mammals could provide a simple, portable, and non-invasive diagnostic tool which would help assess respiratory health and temporal changes in both, dolphins under human care and in wild stranded animals.

5.7. Acknowledgements

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5.8. List of symbols and abbreviations

- $\%VC_{PEF}$ = Percent of the expired VC at the peak expiratory flow
- BAL = Bronchoalveolar lavage
- CT = Computed tomography
- $FEF_{25\%-75\%}$ = Respiratory flow measured between 25% and 75% of the expired vital capacity, or mid forced expiratory flow
- FEV_1 = Forced expiratory volume in the first second
- FVC = Forced vital capacity
- M_b = Body mass
- PEF = Peak expiratory flow
- PFT = Pulmonary function testing
- SL = Straight length
- STPD = Standard temperature pressure dry conditions
- $s\dot{V}$ = Mass specific respiratory flow
- $s\dot{V}_{exp}$ = Specific expiratory flow
- $s\dot{V}_{insp}$ = Specific inspiratory flow
- sV_T = Mass specific tidal volume
- sV_{Texp} = Mass specific expiratory tidal volume
- sV_{Tinsp} = Mass specific inspiratory tidal volume
- TBFVL = Tidal breathing flow-volume loop
- T_{exp} = Expiratory duration
- T_{insp} = Inspiratory duration
- TLC = Total lung capacity
- T_{tot} = Total breath duration
- $\dot{V}$ = Respiratory flow
- VC = Vital capacity
- $\dot{V}_{exp}$ = Expiratory flow
- $\dot{V}_{insp}$ = Inspiratory flow
- V_T = Tidal volume
- V_{Texp} = Expiratory tidal volume
- V_{Tinsp} = Inspiratory tidal volume

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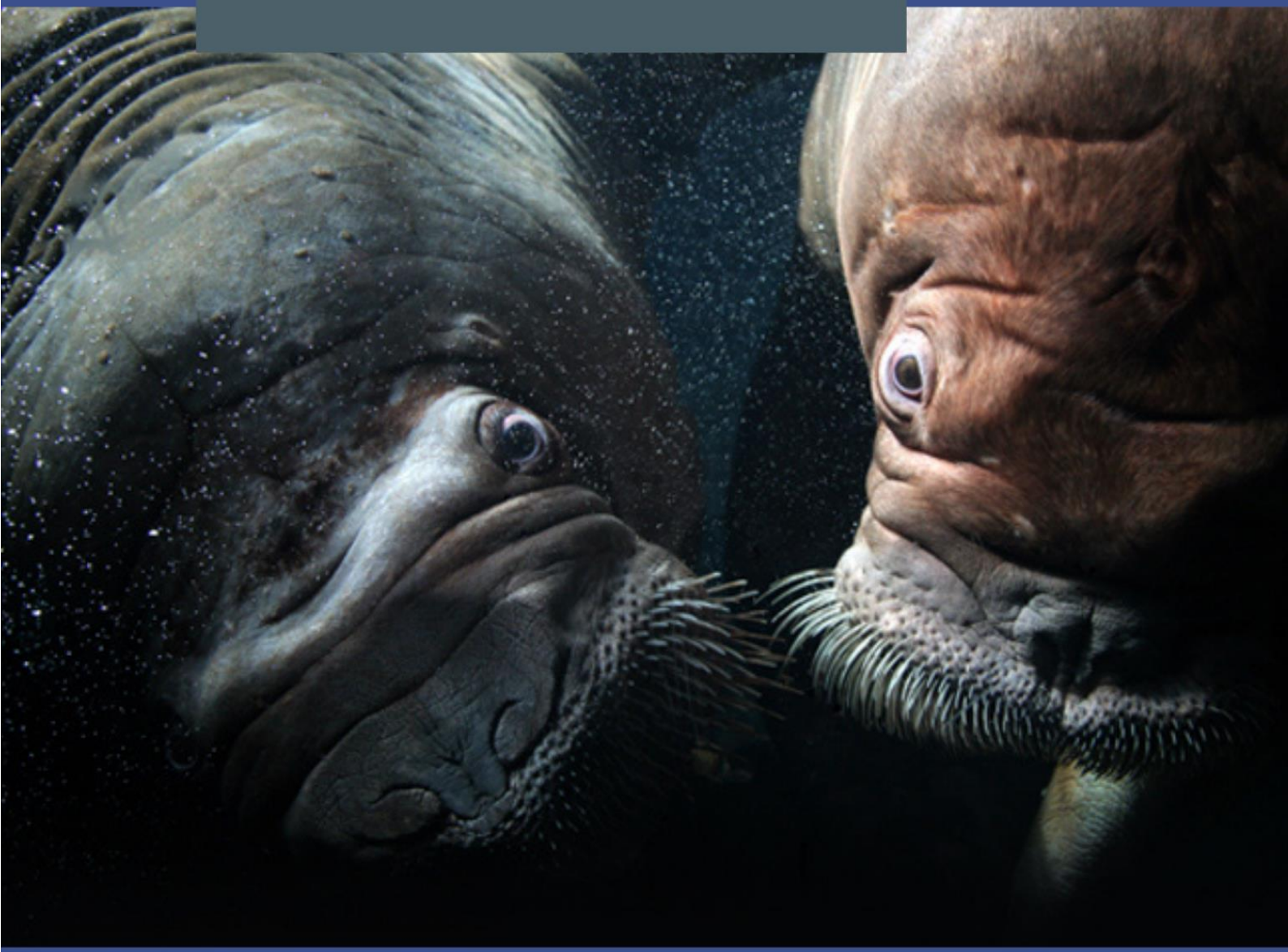
6.Chapter III: Metabolic rates in the Pacific walrus



Subsurface swimming and stationary diving are metabolically cheap in adult Pacific walruses (*Odobenus rosmarus divergens*)

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Valencia

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6. Chapter III: Metabolic rates in the Pacific walrus

6.1. Abstract

Walruses rely on sea-ice to efficiently forage and rest between diving bouts while maintaining proximity to prime foraging habitat. Recent declines in summer sea ice have resulted in walruses hauling out on land where they have to travel farther to access productive benthic habitat while potentially increasing energetic costs. Despite the need to better understand the impact of sea ice loss on energy expenditure, knowledge about metabolic demands of specific behaviours in walruses is scarce. In the present study, 3 adult female Pacific walruses (*Odobenus rosmarus divergens*) participated in flow-through respirometry trials to measure metabolic rates while floating inactive at the water surface during a minimum of 5 min, during a 180-second stationary dive, and while swimming horizontally underwater for ~90 m. Metabolic rates during stationary dives (3.82 ± 0.56 l O_2 min^{-1}) were lower than those measured at the water surface (4.64 ± 1.04 l O_2 min^{-1}), which did not differ from rates measured during subsurface swimming (4.91 ± 0.77 l O_2 min^{-1}). Thus, neither stationary diving nor subsurface swimming resulted in metabolic rates above those exhibited by walruses at the water surface. These results suggest that walruses minimize their energetic investment during underwater behaviours as reported for other marine mammals. Although environmental factors experienced by free-ranging walruses (e.g., winds or currents) likely affect metabolic rates, these results provide important information for understanding how behavioural changes affect energetic costs and can be used to improve bioenergetics models aimed at predicting the metabolic consequences of climate change on walruses.

6.2. Introduction

The Pacific walrus (*Odobenus rosmarus divergens*) inhabits a region of the Arctic that is experiencing some of the most rapid loss of summer sea ice (Laidre et al., 2015; Markus et al., 2009). The sea ice is a critical habitat for this species as it provides a location for resting between foraging bouts, and immediate access to their benthic offshore foraging habitat (Fay, 1982). The sea ice platform is also used for breeding, and females and young walruses usually haul out onto sea ice throughout the year, while adult males haul out on sea ice primarily during winter and spring (Fay, 1982). In recent years, sea ice has retreated beyond the continental shelf in the eastern Chukchi Sea in the fall (Markus et al., 2009). In response, female and young walruses are hauling out in large numbers on land during the summer where they either forage for nearshore prey, or travel farther to more productive benthic habitats in offshore areas (Jay et al., 2012). Walruses with only access to land in the summer spend more time in water while not foraging, and less time hauled out resting as compared with walruses with access to sea ice habitats (Jay et al., 2017). In addition, walruses hauling out on land on the northwest coast of Alaska coast have been documented to migrate to the coast of northern Chukotka in September and October when sea ice is sometimes preserved (Jay et al., 2012). This migration occurs with minimal foraging activity but provides the opportunity to maintain offshore locations (Jay et al., 2012). Migration and foraging travel to maintain access to feeding habitat may increase energetic costs while decreasing energy intake, which could have implications on individual health, survival, and breeding success (MacCracken, 2012). For instance, female walruses increase body size and lipid deposition during pregnancy to support early lactation, but only 27% of caloric requirements during lactation are met by body reserves (Noren et al., 2014). Thus, foraging during lactation is critical for calf survival, and walrus population dynamics are known to be sensitive to successful calf survival (Udevitz et al., 2013). Furthermore, projections of female walrus behaviour with continued sea ice loss suggest that these activity patterns (less time invested on resting and foraging) will become more common in the future (Udevitz et al., 2017).

Understanding the implications of changing behavioural patterns for walrus energetic balance requires information on the energetic demands or metabolic rate associated with individual behaviours. Satellite radio tags deployed on walruses can record location, pressure and conductivity that identify whether a walrus is likely in water foraging or not foraging or hauled out (Fischbach and Jay, 2016; Jay et al., 2006). This

information was recently used in combination with estimated metabolic rates of different behaviours to develop a bioenergetic model for female walruses that allowed prediction of how changes in behaviour in response to sea ice loss or other factors might affect walrus energetic costs. However, model outcomes were most sensitive to estimates of the cost of activity in water (Noren et al., 2012; Udevitz et al., 2017), which has not been directly measured in adult walruses. Data on metabolic rates of walruses are currently limited to a study that reported field metabolic rate (FMR) in two free-ranging adult males (Acquarone et al., 2006), and a study that measured metabolic rates of juveniles while resting in water or during horizontal subsurface swimming (Rosen, 2020). FMR in the two free-ranging adult male Atlantic walruses was measured using doubly-labelled water, which provided estimates of the total energetic costs, but not the energetic costs associated with individual behaviours (Acquarone et al., 2006). More recently, Rosen (2020) reported the first data of activity-associated energetic costs in two trained juvenile walruses. Although these first studies have provided important information to help understand the energetic requirements in this species, we still lack direct measurements of the metabolic cost of specific behaviours for adults.

To increase our understanding about the energetic expenditure in the walrus, the present study aimed to measure the metabolic rate during three different in water behaviours using flow-through respirometry. Three adult female Pacific walruses were trained to perform the experimental procedures for measuring the O_2 consumption rate ($\dot{V}O_2$, l O_2 min^{-1}) while: 1) inactive floating at the water surface (*surface*), and during 2) shallow stationary dives (*stationary*), and 3) horizontal subsurface swimming (*swim*). The initial hypotheses were that the $\dot{V}O_2$ would be lower for stationary dives and subsurface swimming compared to periods while floating at the surface as previously reported for other pinniped species (Castellini et al., 1992; Fahlman et al., 2013; Hurley and Costa, 2001; Reed et al., 1994; Sparling and Fedak, 2004), and that the $\dot{V}O_2$ would be higher during subsurface swimming than that measured during stationary dives.

6.3. Materials and methods

6.3.1. Study subjects and training methodology

Three adult (not spayed) female Pacific walruses [*Odobenus rosmarus divergens* (Illiger, 1815)] born in 2003 and housed at the Oceanogràfic (Valencia, Spain), participated in the present study. Prior to initiating the data collection, the animals

underwent 7 months of desensitization to the respirometry equipment and were trained to perform the different experiments using operant conditioning. This allowed for data collection in a relaxed physiological state, where the animals were free to decide their participation in each experimental trial and could withdraw at any point. The health of the walruses was assessed daily, and all experiments were approved by the Animal Care and Welfare Committee of Fundación Oceanogràfic de la Comunitat Valenciana (Animal Care number: OCE-1-18), and the Animal Care and Use Committee of the U.S. Geological Survey Alaska Science Centre (Review code 2020-01).

6.3.2. General procedures and experimental variables

Data collection took place between August 2018 and November 2019. No data were collected during the reproductive season (from around February to March in the study subjects), due to low interest in participation of the individuals. Body mass (M_b, kg) was measured before the first feeding in the morning the same week of the metabolic trial (± 0-4 days from data collection) and ranged from 682 to 1035 kg (n = 3, average ± standard deviation [s.d.] = 842 ± 116 kg) during the overall experimental period. Ambient pressure, temperature and humidity were measured before commencing each experimental trial (thermometer and hygrometer OH513 Oh Haus & Co), and averaged, respectively, 102.0 ± 0.6 kPa (range = 100.9-103.3 kPa,), 19.1 ± 2.8°C (range = 13.0-25.0°C), and 69 ± 7% (range = 53-84%), during data collection period. The animals were postabsorptive during the metabolic measurements and had not had a meal for at least 15h prior to initiating the experimental trials, but some food reinforcement was provided during the last minute of most respirometry trials to help reinforce the required experimental behaviour. The regular diet included a mixture of fish (capelin and herring) and molluscs (squid and mussels) supplemented with vitamins. The estimated energy intake (kJ_{est}, kJ) during each metabolic trial was calculated using the measured amount of consumed food (kg) and the measured energetic content for each type of food (kJ kg^{-1}). This energetic content was estimated in-house through bomb calorimetry of random samples of the food items that arrived at the aquarium.

All metabolic measurements were conducted in a 3 m deep seawater pool with a total water volume of 267 m^3. The water temperature at the facility was measured daily and ranged from 12.9 to 18.6°C (average = 15.9 ± 1.0°C) during the overall experimental period. Data collection was performed using an open flow respirometry system early described in the General methodology section. The animals were trained to surface into a

respirometry dome and keep breathing inside the respirometer while floating in a relaxed and inactive state (see Figure 3.6 in the General methodology section). The walruses showed some corporal movement while maintaining the required position inside the respirometer. Consequently, the animals were not quiescent throughout the whole duration of the experimental trials. The measured changes in O_2 and CO_2 content of the air exiting the respirometry dome relative to atmospheric air were used to estimate the $\dot{V}O_2$ and the CO_2 production rate ($\dot{V}CO_2$, l CO_2 min^{-1}) for each experimental trial. The estimated $\dot{V}O_2$ was used to define the metabolic rate for the three in water behaviours: 1) while floating at the water surface (metabolic rate at the surface [$MR_{Surface}$, l O_2 min^{-1}]), and during 2) shallow stationary dives (diving metabolic rate during stationary dives [$DMR_{Stationary}$, l O_2 min^{-1}]), or 3) subsurface swimming dives (diving metabolic rate while swimming [DMR_{Swim}, l O_2 min^{-1}]).

The $MR_{Surface}$ was estimated from at least 5 minutes of data collection while the walrus remained calmly floating inside the respirometer. For $DMR_{Stationary}$ measurements, the walrus was directed to submerge to a fixed point at the bottom of the pool and to remain stationary while holding its breath for at least 3 minutes. For the DMR_{Swim} estimation, the animal swam back and forth between two underwater target poles that were positioned approximately ~7 m apart, until reaching ~90 meters of horizontal subsurface swimming. The walruses swam at a depth of ~1.5 m, and all trials were timed to calculate the swimming velocity. These methods are similar to the swimming trials conducted with juvenile walruses in Rosen (2020). To determine either $DMR_{Stationary}$ or DMR_{Swim}, the walrus was guided to surface inside the respirometry dome at completion of the dives to measure the gas exchange during the post-dive recovery period. The post-dive recovery period ended when the $\dot{V}O_2$ and $\dot{V}CO_2$ had returned to similar values recorded during the last portion of $MR_{Surface}$ measurements, and remained stable for at least 1 min. Exploratory trials prior to data collection showed that the $\dot{V}O_2$ and $\dot{V}CO_2$ returned to steady values within the range (± s.d.) of those measured during $MR_{Surface}$ trials between ~4 min and ~6 min for the stationary dives and subsurface swimming, respectively (see an example in Figure 6.1). Therefore, to ensure the inclusion of the entire physiological recovery for all the trials, the post-dive recovery period was extended respectively to 5 minutes and 7 minutes for the stationary dives and subsurface swimming.

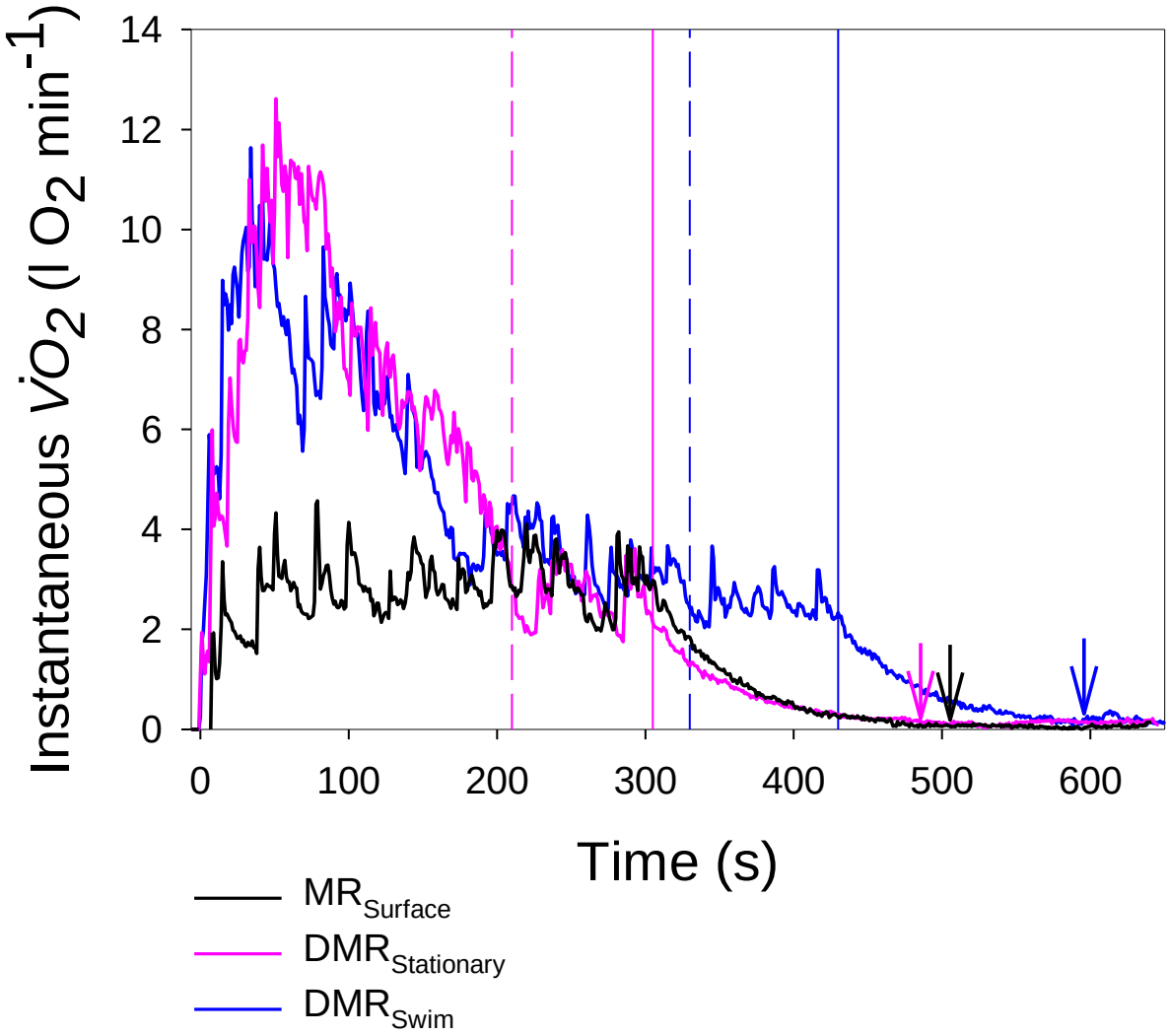

Figure 6.1. An example representation of oxygen consumption measurements for one Pacific walrus during three in water behaviours.
Measured instantaneous O_2 consumption rate ($\dot{V}O_2$) for one adult female Pacific walrus (*Odobenus rosmarus divergens*, Animal ID = 26005388) during $\dot{V}O_2$ measurements while floating at the water surface ($MR_{Surface}$), and during post-dive $\dot{V}O_2$ measurements for stationary dives ($DMR_{Stationary}$) and subsurface swimming (DMR_{Swim}). For the three experimental trials, the walrus entered the dome at time = 0 s. For the post-dive recovery period of both underwater experiments, the dashed vertical lines represent the period when the $\dot{V}O_2$ was similar to that obtained during the last portion (~2 minutes) of $MR_{Surface}$ measurements, while the solid vertical lines indicate the time when the walrus left the respirometer ($DMR_{Stationary}$: pink lines; DMR_{Swim}: blue lines). The total volume of O_2 consumed for each trial was computed by integrating the instantaneous $\dot{V}O_2$ from the walrus entered the dome until the O_2 returned to ambient values (instantaneous $\dot{V}O_2$ ~0) which is indicated by the arrows ($MR_{Surface}$: black; $DMR_{Stationary}$: pink; DMR_{Swim}: blue). For both underwater behaviours, the $DMR_{Stationary}$ and DMR_{Swim} were computed as the total volume of consumed O_2 divided by the entire dive cycle (dive duration + post-dive recovery duration). As an example, the represented $DMR_{Stationary}$ was estimated by integrating the $\dot{V}O_2$ until the position of the pink arrow and diving the obtained O_2 volume by the dive duration (180s) plus the duration of the measurement (304 s, i.e., when the walrus left the respirometer).

6.3.3. Respiratory gas exchange measurements

The open-flow respirometry system (see Figure 3.7 in the General methodology section) consisted of a vacuum pump (FlowKit Mass Flow Generator, FK-500-1, Sable Systems Int., Las Vegas, NV, USA) pulling air through a floating transparent Plexiglas dome of 120 cm internal diameter (i.d.) via an 800 cm length and 4.5 cm i.d. plastic corrugated tube. The dome was made buoyant by attaching polyethylene foam to the base. A flow-through rate of 500 l min^{-1} of air assured that the O_2 and CO_2 were maintained > 19% and < 2%, respectively. The O_2 and CO_2 content were measured using a fast-response gas analyser (Gemini Respiratory Gas Analyzer, part no. 14-1000, CWE Inc., Allentown, PA, USA), which pulled a subsample of the outlet air from the corrugated tube at a flow rate of 200 ml min^{-1} via a 310 cm length and 2 mm i.d. firm walled, flexible tubing. This flexible tubing was attached to a hydrophobic filter (13 mm i.d.), followed by a 60 cm length and 1.5 mm i.d. Nafion© sample line connected to the gas analyser. The gas analyser was routinely calibrated using ambient air and a commercial gas mixture (5% O_2, 5% CO_2, and 90% N_2; UN1956 Air Liquide, USA). Both, respiratory gas concentrations and air flow rates were captured at 400 Hz using a data acquisition system (Powerlab 8/35, ADInstruments, Colorado Springs, CO, USA), and displayed on a laptop computer running Labchart (v. 8.1, ADInstruments). As previously described in the General methodology section, a simultaneous CO_2 and O_2 dilution test was conducted to evaluate the system for possible leaks, and to the assess the accuracy in measured O_2 and CO_2 concentrations (Fahlman et al., 2008; Fedak et al., 1981), which were within 6% of estimated values. The effective volume of the system was 465 l, which resulted in a time constant of 0.93 min (Bartholomew et al., 1981), while the time required to reach a 95% fractional transformation to a new steady state was 167 s (2.79 min) or 3 times the time constant (see General Methodology section; Bartholomew et al., 1981; Fahlman et al., 2007).

6.3.4. Data processing and criteria for data inclusion

The gas analyser baseline drift during an experimental trial was corrected by measuring the ambient air concentrations at the end of the trial. As previously described in the General methodology section, measured flow rates were corrected to standard temperature and pressure dry conditions (STPD, Quanjer et al., 1993), and were multiplied by measured gas concentrations to calculate the instantaneous $\dot{V}O_2$ and $\dot{V}CO_2$, which were corrected for variation in the respiratory exchange ratio (Withers, 1977).

The following criteria were used to include a metabolic trial in the analysis. For all trials, the walrus had to remain calm with all breaths taken inside the respirometer. For experiments while floating at the surface, the walrus had to remain at least 5 min inside the respirometer. For diving trials, the walrus had to 1) complete the pre-determined dive duration of 3 min for the stationary dives or the horizontal swimming distance of ~90 m for the subsurface swimming, and 2) remain inside the respirometer at least 5 min for stationary dives or 7 min for subsurface swimming during the post-dive recovery.

6.3.5. Metabolic rates calculations and conversions

To determine the total volume of O_2 consumed during each experimental trial, the instantaneous $\dot{V}O_2$ was integrated over the period from when the walrus surfaced into the respirometer, until the O_2 had returned to ambient levels after leaving the respirometer (Figure 6.1). Obtained O_2 volume was then divided by the entire measurement period for the $MR_{Surface}$ trials, and by the total entire dive cycle or the dive time plus the post-dive recovery time for the $DMR_{Stationary}$ and DMR_{Swim} trials (see an example in Figure 6.1).

The Kleiber ratio was calculated for each behaviour by dividing the measured $\dot{V}O_2$ by the predicted basal metabolic rate (BMR_{est}, l O_2 min^{-1}) for a similarly sized terrestrial mammal (BMR_{est} = 0.0093 $M_b^{0.75}$ l O_2 min^{-1}; Kleiber, 1975). The $\dot{V}CO_2$ for each behaviour was computed as described for $\dot{V}O_2$ and used to determine the respiratory exchange ratio (RER, $\dot{V}CO_2$ divided by $\dot{V}O_2$). The mass-specific metabolic rate was calculated for each experimental procedure (floating at the surface: $sMR_{Surface}$; stationary dives: $sDMR_{Stationary}$; subsurface swimming: $sDMR_{Swim}$; ml O_2 kg^{-1} min^{-1}) by dividing the metabolic rate by M_b and assuming isometry. Measured metabolic rates and mass-specific conversions for the different experimental procedures were converted into daily energetic requirements as: metabolic rate (kJ day^{-1}) = [(16.218 + 4.716 · RER) · $\dot{V}O_2$ · 60 · 24]. This formula is based on the assumptions that protein catabolism is negligible, while carbohydrates and lipids liberate 20.93 and 19.55 kJ per 1 litre of consumed O_2, respectively (see Table 17 in Jungas et al., 1992). The respiratory frequency (f_R, breaths min^{-1}) was computed for each behaviour as the number of observed breaths by the total time of the measurement period. The calculated aerobic dive limit (cADL, min) was estimated by dividing the estimated total body O_2 stores by the obtained DMR_{Swim} (Butler, 2006). The calculations to yield the total accessible body O_2 stores during diving are detailed in the Supplementary materials and methods section and were performed by

summing the oxygen capacity of different tissues (blood, muscle, and lungs), and by estimating the level of O_2 utilization from each tissue during the dive (Kooyman, 1989).

6.3.6. Statistical analysis

The initial analysis aimed to evaluate the relationship between measured $MR_{Surface}$, $DMR_{Stationary}$, DMR_{Swim} and the experimental covariates (M_b, and water temperature as continuous variables) using linear models (*lm* function in R; R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, version 3.6.1, 2019). A backward stepping procedure implemented by the R-function *step* was used to identify whether M_b and water temperature were important in affecting metabolic rates, based on the combination of variables that had the lowest Akaike Information Criterion (AIC). The importance of the covariates in the final models were verified by performing the log-likelihood ratio test to confirm that the full model was better than a model containing just the intercept. Measured $MR_{Surface}$, $DMR_{Station}$, and DMR_{Swim} were then compared using an ANCOVA F test that included the experiment as a fixed factor, and the covariates identified from the backward stepping procedure as independent variables. The animal identification (ID) was initially included as a random effect to account for the correlation between repeated measurements on the same individual (Littell et al., 1998), but was removed from the models due to its strong correlation with M_b. A Tukey HSD Post Hoc test was used to determine whether $DMR_{Station}$, and DMR_{Swim} were different from $MR_{Surface}$. For all analyses M_b and $\dot{V}O_2$ were transformed using the base 10 logarithm. Homoscedasticity and normality were assessed through evaluation of residual plots. In the present study, p-values ≤ 0.05 were considered significant, and data are presented as the mean ± s.d.

6.4. Results

6.4.1. Total and selected trials, and experimental variables

The three female Pacific walruses participated in a total of 108 respirometry experimental trials including training and sampling sessions (2018: n = 20 trials, 2019: n = 88 trials). From the total experimental trials, 5 trials for each animal and behaviour (n = 45 for all animals and behaviours) passed the selection criteria to be included in the analysis. For selected trials, the measured M_b ranged from 688 kg to 1030 kg (835 ± 112 kg, Table 6.1), and the kJ_{est} ranged from 96 kJ to 6238 kJ (n = 44) which represented

0.1%-5.5% of the established total daily energy intake of the animals for the dates of data collection (Table 6.1).

Table 6.1. Body mass and estimated energy intake for each Pacific walrus during metabolic measurements.

Animal ID	M_b (kg)	kJ_{est} (kJ)	$\%kJ_{tot}$
26005388	705 ± 9 (688-721)	1236 ± 2108 (100-6238)[15]	1.2 ± 1.9 (0.1-5.5)
26005389	975 ± 25 (950-1030)	1586 ± 2445 (96-6238)[14]	1.4 ± 2.1 (0.1-5.5)
26005390	825 ± 7 (813-834)	207 ± 74 (100-339)[15]	0.2 ± 0.1 (0.1-0.3)

For trials included in the analysis (n = 45), animal identification (ID) of each participating adult female Pacific walrus (*Odobenus rosmarus divergens*, n = 3) and average (± s.d) and ranges of body mass (M_b), estimated energy intake during the experimental procedure (kJ_{est}), and percentage of energy intake in relation to the total daily intake for the days of the experiments ($\%kJ_{tot}$). Superscripts in the kJ_{est} column indicate the number of trials where the animals were provided with food during the last minute of the procedure.

For trials included in the analysis, the measured humidity outside and inside the dome were, respectively, 71 ± 7% (54 – 82%) and 69 ± 7% (53-81%), while the ambient pressure and air temperature at the facility housing the animals were 101.8 ± 0.4 kPa (100.9-102.6 kPa) and 19.2 ± 2.1°C (15.4-23.1°C), respectively. The water temperature for selected trials averaged 15.4 ± 1.0°C (Table 6.2).

Table 6.2. Trial information and water temperature for each metabolic experiment in Pacific walruses.

Experiment	Dive duration (min)	Data collection (min)	Water T (°C)
Floating at the water surface	-	5.1-7.0	12.9-17.0
Stationary dive	3.0-3.3	5.0-5.6	14.3-17.1
Subsurface swimming	1.2-1.8	7.0-7.5	14.1-16.9

For trials included in the analysis (n = 15 for each metabolic experiment), ranges of dive duration for underwater behaviours, duration of data collection, and water temperature (Water T) for metabolic trials with three participating adult female Pacific walruses (*O. rosmarus divergens*) while floating at the water surface, and after performing stationary dives and subsurface swimming dives.

The backward stepping procedure showed that the elimination of the covariate "water temperature" from the model increased the AIC for $MR_{Surface}$ trials ($\Delta AIC = 0.313$) and decreased AIC for both underwater behaviours by less than 2 units ($DMR_{Stationary}$: $\Delta AIC = 1.28$; DMR_{Swim}: $\Delta AIC = 1.74$). For all experiments, the coefficient for water temperature was not significant (Wald's t test, *p*-value: $MR_{Surface} = 0.18$; $DMR_{Stationary} = 0.45$; $DMR_{Swim} = 0.65$). Because water temperature either did not improve model fit or had only a minor, non-significant effect on metabolic rate, this covariate was not included

it in models comparing metabolic rates between behaviours. Alternatively, the log-likelihood ratio test showed that inclusion of M_b improved the fit of the model compared to the intercept-only model ($p < 0.05$ for all experiments, Table 6.3). Therefore, only M_b was included in the ANCOVA F test comparing metabolic rates between behaviours. For each studied behaviour, measured M_b, $\dot{V}CO_2$, f_R, and RER are reported in Table S1 in the supplementary tables section, while measured $\dot{V}O_2$ for each participating walrus and behaviour is reported in terms of daily energetic requirements in Table S2.

Table 6.3. Statistical results evaluating the relationship between metabolic rate and body mass for three in-water behaviours in Pacific walruses.

Experiment	χ^2	*p*-value	β_0	$\log_{10}(M_b)$	R^2
$\text{Log}_{10}(\text{MR}_{\text{Surface}})$	13.73	<0.05	-2.91 ± 0.81	1.22 ± 0.28	0.60
$\text{Log}_{10}(\text{DMR}_{\text{Stationary}})$	7.96	<0.05	-1.55 ± 0.71	0.73 ± 0.24	0.41
$\text{Log}_{10}(\text{DMR}_{\text{Swim}})$	27.60	<0.05	-2.61 ± 0.40	1.13 ± 0.14	0.84

For each metabolic behaviour (n = 15 per each metabolic experiment) measured in three adult female Pacific walruses (*O. rosmarus divergens*), linear models included log10-transformed ($\log_{10}$) metabolic rate measured in l O_2 min^{-1} (floating at the water surface: $MR_{Surface}$; stationary dives: $DMR_{Stationary}$; subsurface swimming: DMR_{Swim}) as the dependent variables, and $\log_{10}$ body mass (M_b, measured in kg) as the covariate. The results show the χ^2 for the log-likelihood ratio test and the obtained *p*-value when comparing the model including M_b with the model including only the intercept. The intercept (β_0) the coefficient for $\log_{10}(M_b)$ together with the standard error, and the r-squared (R^2) resulting from the final models including M_b are additionally reported.

6.4.2. Metabolic rate while floating at the water surface

The average duration for $MR_{Surface}$ trials was 5.7 ± 0.6 min (Table 6.2). The average $MR_{Surface}$ (4.64 ± 1.04 l O_2 min^{-1}, Table 6.4) was higher than the predicted BMR_{est} for a similarly sized terrestrial mammal (1.55 ± 0.17 l O_2 min^{-1}, Figure 6.2), and the Kleiber ratio was higher than 1 for all animals (3.0 ± 0.5; range = 2.3-3.9). The $MR_{Surface}$ increased with M_b with a mass-exponent close to 1 (Table 6.3 and Figure 6.2).

6.4.3. Metabolic rates during stationary dives and subsurface swimming

The average breath-hold duration for stationary dives and subsurface swimming were 3.2 ± 0.1 min and 1.6 ± 0.2 min, respectively (Table 6.2), with an average swimming velocity of 1.0 ± 0.1 m s^{-1} (range = 0.9-1.3 m s^{-1}) during the subsurface swimming. The average post-dive recovery duration for $DMR_{Stationary}$ trials was 5.2 ± 0.1 min, and 7.2 ± 0.1 min for DMR_{Swim} trials (Table 6.2). The $MR_{Surface}$ differed from those measured during the underwater behaviours (One-way ANCOVA F-test, F [2, 41] = 16.48, $p < 0.05$, Figures 6.2 and 6.3), where the $DMR_{Stationary}$ (3.82 ± 0.56 l O_2 min^{-1}, Table 6.4) was

significantly lower as compared with $MR_{Surface}$ (Tukey HSD test, Mean difference = -0.071, 95% CI [-0.117, -0.026], $p < 0.05$), while DMR_{Swim} (4.91 ± 0.77 l O_2 min^{-1}, Table 6.4) and $MR_{Surface}$ did not differ (Tukey HSD test, Mean difference = 0.034, 95% CI [-0.011, 0.080], $p < 0.05$). The Kleiber ratio, was higher than 1 for all animals and underwater behaviours (Figure 6.2) and averaged 2.5 ± 0.3 (range = 1.9-2.8) for stationary dives, and 3.2 ± 0.3 (range = 2.6-3.5) for subsurface swimming. For an 835 kg adult female walrus the cADL was 7.7 min.

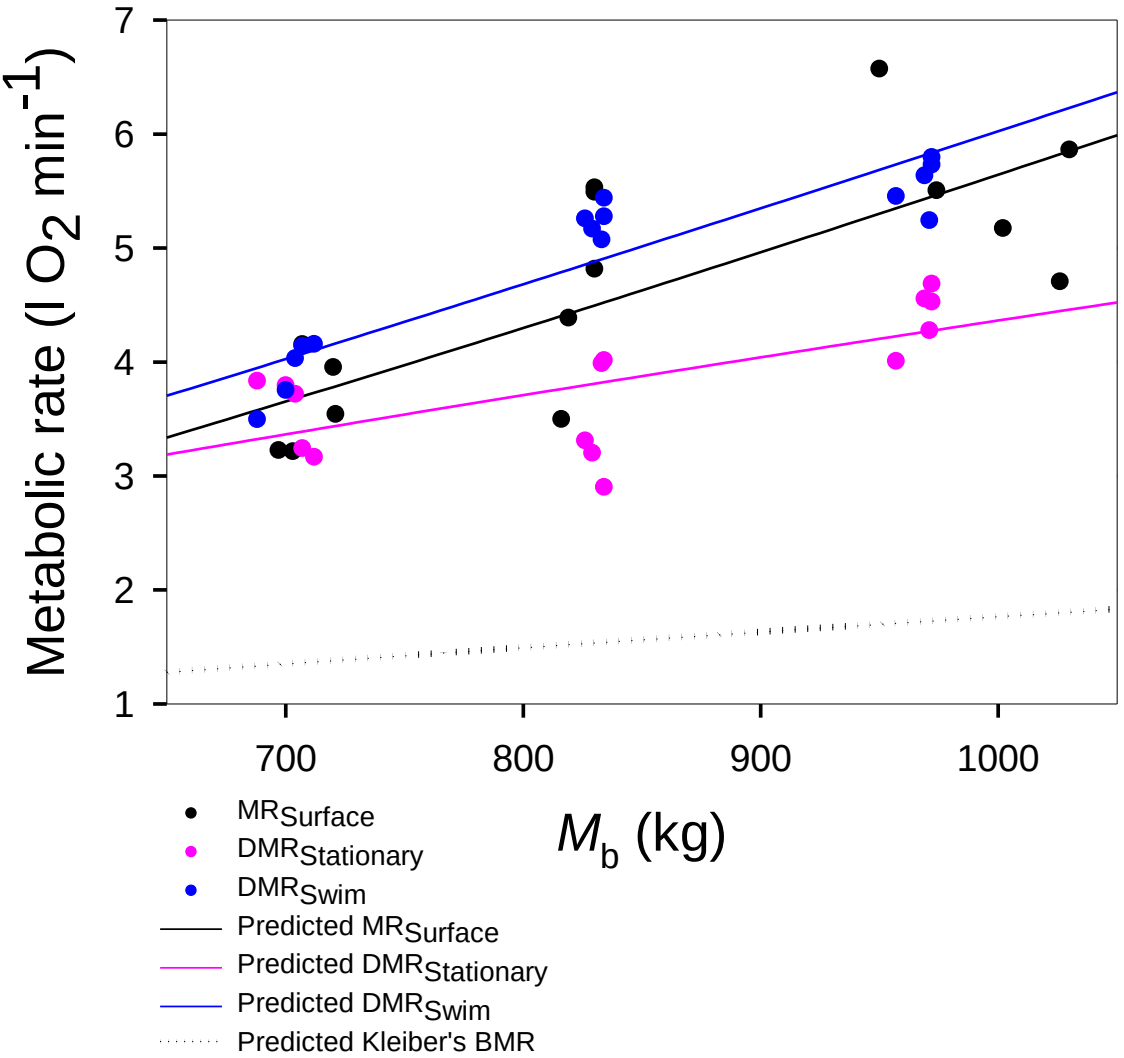

Figure 6.10. Measured metabolic rates in relation to body mass for three in-water behaviours in walruses.

Relationship between measured metabolic rates and body mass (M_b) from three adult female Pacific walruses (*O. rosmarus divergens*) participating in experimental trials (n = 5 per each animal and experimental procedure) while floating at the water surface ($MR_{Surface}$), and during stationary dives ($DMR_{Stationary}$) and subsurface swimming (DMR_{Swim}). The solid lines represent the predicted metabolic rates (l O_2 min^{-1}) obtained from the results of the linear models reported in Table 6.3 ($MR_{Surface} = 0.00123 \cdot M_b^{1.22}$; $DMR_{Stationary} = 0.0282 \cdot M_b^{0.73}$; $DMR_{Swim} = 0.00245 \cdot M_b^{1.13}$). The dotted line represents the predicted Kleiber's basal metabolic rate (BMR_{est} l O_2 $min^{-1} = 0.00993 \cdot M_b^{0.75}$, Kleiber, 1975).

Table 6.4. Summary of metabolic measurements in Pacific walruses.

ANIMAL ID	$MR_{Surface}$ ($l\ O_2\ min^{-1}$)	$sMR_{Surface}$ ($ml\ O_2\ kg^{-1}\ min^{-1}$)	$DMR_{Stationary}$ ($l\ O_2\ min^{-1}$)	$sDMR_{Stationary}$ ($ml\ O_2\ kg^{-1}\ min^{-1}$)	DMR_{Swim} ($l\ O_2\ min^{-1}$)	$sDMR_{Swim}$ ($ml\ O_2\ kg^{-1}\ min^{-1}$)
26005388	3.62 ± 0.42	5.09 ± 0.57	3.55 ± 0.32	5.04 ± 0.41	3.92 ± 0.28	5.57 ± 0.34
26005389	5.57 ± 0.70	5.61 ± 0.86	4.41 ± 0.27	4.60 ± 0.26	5.57 ± 0.23	5.76 ± 0.22
26005390	4.74 ± 0.85	5.74 ± 0.98	3.48 ± 0.50	4.25 ± 0.61	5.24 ± 0.14	6.31 ± 0.16
Overall range	3.22-6.57	4.29-6.92	2.90-4.69	3.53-5.40	3.50-5.80	5.08-6.52

For each participating female (Animal ID) Pacific walrus (*O. rosmarus divergens*, n = 3) and behaviour, average (± s.d) of measured metabolic rate (floating at the water surface: $MR_{Surface}$; stationary dives: $DMR_{Stationary}$; subsurface swimming: DMR_{Swim}) and mass-specific metabolic rate (floating at the water surface: $sMR_{Surface}$; stationary dives: $sDMR_{Stationary}$; subsurface swimming: $sDMR_{Swim}$). N = 5 for all experiments with each individual. Overall ranges for each behaviour are also reported.

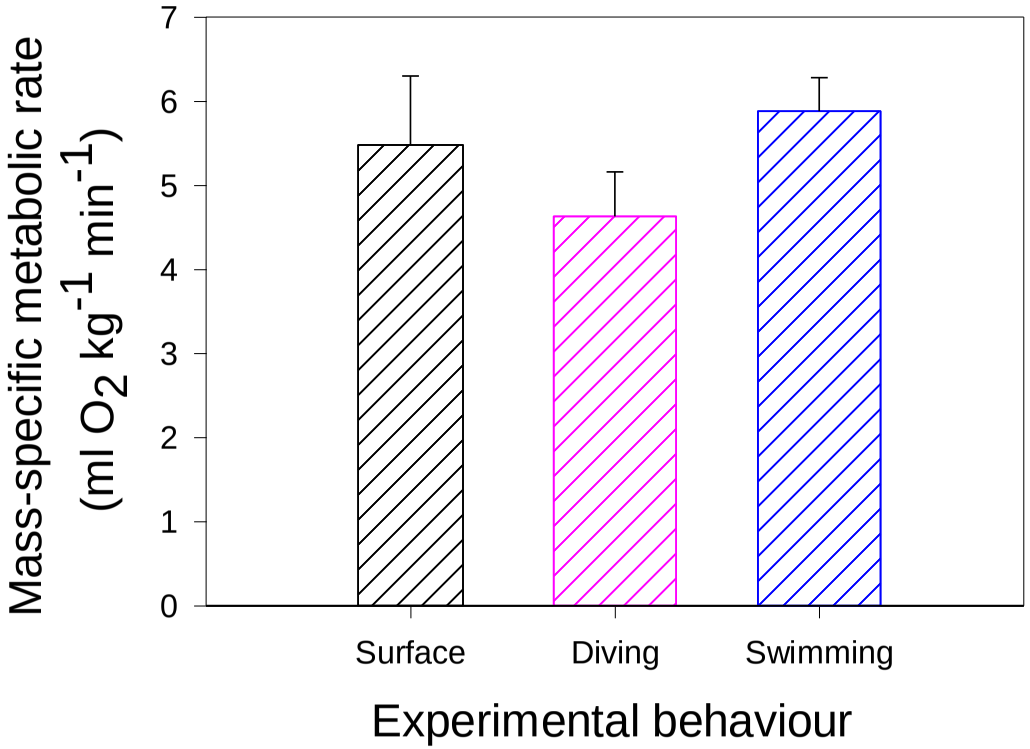

Figure 6.17. Energetic investment of three Pacific walruses during different in water behaviours.

For the three participating adult female Pacific walruses (*O. rosmarus divergens*), average (± s.d.) mass-specific metabolic rates measured using flow-through respirometry while floating at the water surface (surface, black), and during stationary dives (diving, pink) and subsurface swimming (swimming, blue). N = 15 trials per metabolic activity.

6.5. Discussion

This study reports the first metabolic rate estimates from adult individuals of the largest pinniped species yet studied while floating at the water surface and during stationary dives and subsurface swimming in a controlled environment. The results from the three adult female Pacific walruses are similar to those previously reported for post-absorptive adult pinnipeds, where measured resting metabolic rate (RMR) was 2-3 times greater as compared with BMR_{est} (Dassis et al., 2012; Fahlman et al., 2008; Fahlman et al., 2013; Hurley and Costa, 2001; Sparling and Fedak, 2004). The range for $sMR_{Surface}$ for the adult female walruses in the present study overlapped and exceeded the upper end of $sMR_{Surface}$ measured for juvenile walruses (Rosen, 2020). The initial hypotheses were that the $DMR_{Stationary}$ and DMR_{Swim} would be lower than the $MR_{Surface}$, and that subsurface swimming would be more energetically costly than stationary dives. However, the results indicated that $DMR_{Stationary}$ was lower than $MR_{Surface}$ and DMR_{Swim}, while DMR_{Swim} was similar to $MR_{Surface}$. These results agree with previous studies in pinnipeds that showed a lower diving metabolic rate (DMR) for inactive dives compared to RMR at the surface (Castellini et al., 1992; Hurley and Costa, 2001), and other studies reporting a DMR_{Swim} lower or similar to RMR (Castellini et al., 1992; Fahlman et al., 2008; Fahlman et al., 2013; Sparling and Fedak, 2004). The results presented in this chapter suggest that both stationary diving and subsurface swimming are not energetically costly for walruses. Obtained $sDMR_{Swim}$ in the present study was lower than that measured in juvenile walruses performing similar subsurface swimming trials (Rosen, 2020), and compared to measured mass-specific FMR for free-ranging adult male walruses (Acquarone et al., 2006). The estimated cADL was within the range of dive durations observed in free-ranging walruses (Acquarone et al., 2006; Born et al., 2005; Gjertz et al., 2001; Wiig et al., 1993).

6.5.1. Metabolic rates while floating at the water surface

Measured $MR_{Surface}$ in the present study was similar to previous studies reporting basal metabolic rate (BMR) or RMR from post-absorptive and desensitized adult pinnipeds, where measured metabolic rates were 2-3 times greater than that predicted by Kleiber's equation for terrestrial mammals (Dassis et al., 2012; Fahlman and Madigan, 2016; Fahlman et al., 2020; Fahlman et al., 2008; Fahlman et al., 2013; Hurley and Costa, 2001; Rosen, 2020; Sparling and Fedak, 2004). While the reasons for the higher measured BMR or RMR in marine mammals as compared with the predictions from Kleiber's

remain unclear, it has been suggested that different methodologies and physiological states of the animals during metabolic measurements may be possible reasons for these differences (Lavigne et al., 1986). Kleiber (1975) defined the standard conditions that a study subject should accomplish to obtain comparable metabolic measurements under basal conditions or BMR: 1) reproductively mature individuals, under 2) post-absorptive state of digestion, 3) thermoneutral conditions, and 4) minimal activity level while awake. In the present investigation, the procedures fulfilled most of these established criteria. The three female walruses were adults, and the experimental procedures took place following an overnight fast. Although a small amount of food was given during the last minute of the trial, this is unlikely to have significantly altered measured metabolic rates considering that it takes approximately 30 minutes to detect a metabolic increase after food ingestion (Rosen and Trites, 1997). The thermoneutral zone for walruses in water is still unknown, and the water temperatures in their natural environment at the Chukchi Sea (temperature range at the surface = from 0 to 10ºC, and at 30 m depth = from -1 to 6ºC, Luchin and Panteleev, 2014) are lower than those measured during selected trials in the present study (range = 12.9-17.1ºC). However, measured metabolic rates were not related to the range of water temperatures during the metabolic trials in the current study. Therefore, due to the lack of information about the thermoneutral range in this species measured $MR_{Surface}$ in the current study should not be considered as estimates of BMR for the walrus.

In addition, the animals were trained to maintain a relaxed and inactive state during metabolic measurements to allow for measuring RMR, which is defined as the metabolic rate measured at rest when other conditions for BMR are not met. However, it is worth mentioning that the measured $MR_{Surface}$ may have been higher than actual RMR because the walruses had to move to maintain their upright position inside the respirometry dome. In fact, juvenile walruses that remained motionless while in a respirometry dome (Rosen, 2020) had slightly lower $sMR_{Surface}$ (range = 73.1-166.1 kJ kg^{-1} day^{-1}) that the adult females in the present study (range = 126.3-203.9 kJ kg^{-1} day^{-1}, Table S2), despite that higher sMR_{Suface} is expected for juvenile individuals due to their higher mass-specific metabolic rates and investment in growth and development (Costa, 1993). Other studies have similarly noted difficulty maintaining the animals motionless when in water (Dassis et al., 2012; Fahlman et al., 2008). For example, in Patagonia sea lions (*Otaria flavescens*) measured RMR on land was 1.4 times lower as compared with

$MR_{Surface}$, which was attributed to movement inside the respirometry dome (Dassis et al., 2012). Still, the movement shown by the participating walruses at the water surface in this investigation may be consistent with the actual behaviour in their natural environment, where it could be difficult to maintain a motionless position at sea surface conditions. Therefore, although the obtained Kleiber ratio for measured $MR_{Surface}$ in the present study was similar to BMR or RMR previously reported for pinnipeds, measured $MR_{Surface}$ in the current study may be elevated as compared with RMR due to observed movement while in the respirometer, but may represent a more applicable approximation of the actual $MR_{Surface}$ for those animals in the wild. In addition, metabolic rates have been reported to scale exponentially with M_b with a mass-exponent of 0.75 (Kleiber, 1975). While in the present study obtained $MR_{Surface}$ closely followed a linear relationship with M_b (see Table 6.3 and Figure 6.2), the limited M_b range may not allow an appropriate analysis to determine whether the relationship is linear or exponential.

6.5.2. Metabolic rates during stationary dives and subsurface swimming

On average, $DMR_{Stationary}$ of the adult female walruses was lower as compared with $MR_{Surface}$ and DMR_{Swim}, which is consistent with previous studies investigating stationary or inactive dives in pinnipeds (Castellini et al., 1992; Hurley and Costa, 2001). For example, in trained California sea lions (*Zalophus californianus*), Hurley and Costa (2001) reported lower $DMR_{Stationary}$ than $MR_{Surface}$ for shallow dives (see Figure 3 in Hurley and Costa, 2001). In freely diving Weddell seals (*Leptonychotes weddellii*), Castellini et al. (1992) reported metabolic rates during short sleep or inactive apneas (<14 min) below $MR_{Surface}$ (see Figure 3 in Castellini et al., 1992). The same authors also reported that the overall DMR_{Swim} during free diving was 1.5 times greater than that observed for sleep apneas of similar duration (Castellini et al., 1992), which is similar to the results from the present study in which DMR_{Swim} was 1.3 times greater than $DMR_{Station}$.

Alternatively, DMR_{Swim} did not differ from $MR_{Surface}$ which agrees with previous studies in pinnipeds that showed a DMR_{Swim} close to or below RMR in freely diving animals (Castellini et al., 1992; Sparling and Fedak, 2004) or in subjects trained to swim vertically (Fahlman et al., 2008; Fahlman et al., 2013). In addition, these studies also reported a decreased DMR_{Swim} with increased dive duration, suggesting that pinnipeds can decrease their energetic investment depending on the dive requirements. This extended diving hypometabolism was first described by Scholander (1940), and was

suggested to be an ability to reduce metabolism by decreasing the activity of different metabolic processes (e.g., digestion, liver and kidney function, etc.), that would increase the dive duration. While in the present study changes in DMR with increased dive duration were not assessed, obtained results suggest an efficient O_2 use during underwater behaviours in the walrus. This physiological capability could explain the reduced $DMR_{Stationary}$ and how measured DMR_{Swim} was similar to $MR_{Surface}$ despite the additional energetic investment during subsurface swimming.

On the other hand, despite the similarity of the procedures during subsurface swimming trials in two juvenile walruses trained to swim horizontally for 110 m, at a depth of ~1.2 m, and at constant velocities (male = 1.6 ± 0.1 m s^{-1}; female = 2.1 ± 0.1 m s^{-1}), the obtained average $sDMR_{Swim}$ (male = 242.9 ± 60.0 J kg^{-1} min^{-1}; female = 336.6 ± 84.4 J kg^{-1} min^{-1}; Rosen, 2020) were up to 2.8 times greater than that measured in the present study (121.3 ± 7.0 J kg^{-1} min^{-1}). However, Rosen (2020) noticed that the estimated cost of transport during subsurface swimming in these juvenile walruses was elevated over to that predicted for a similarly sized marine mammal, which was suggested to be related with non-optimal swimming speed and with the higher underlying maintenance costs of young individuals (Rosen, 2020). Thus, the decreased $sDMR_{Swim}$ reported in the adult female walruses may be partly related with the lower swim speeds (1.0 ± 0.1 m s^{-1}) and distance (90 m) for subsurface swimming trials in the present study, but also to the decrease in mass-specific metabolic rates with age (Kleiber, 1975). In addition, obtained RER ranges for all experimental behaviours (0.81-1.06, see Table S1) were within expected values. While the RER immediately following a dive tends to change dynamically as the blood and tissues are recovering (Fahlman et al., 2008; Reed et al., 1994), the average for RER during trials at the water surface (0.97 ± 0.05) were similar to those obtained during the post-dive recovery period for both underwater experiments (stationary dives = 0.94 ± 0.07; subsurface swimming = 0.94 ± 0.06), and agreed with data reported for resting periods at the surface and for conditioned dives in Steller sea lions (see Table 1 in Fahlman et al., 2008).

When translating the results obtained in this study to free-ranging walruses, we found similar characteristics of subsurface swimming behaviour with those previously reported in free-ranging animals that were associated with travelling or exploratory behaviours (Gjertz et al., 2001; Jay et al., 2001). These dives are reported to be shallow (< 4 m), with most of them lasting < 3 min, and occurring most frequently at the beginning

and ending of foraging trips (Gjertz et al., 2001; Jay et al., 2001). The associated ascent and descent swimming velocity reported for these short dives is ~0.5 m s^{-1}, but exceeds 1 m s^{-1} for longer and deeper dives (Gjertz et al., 2001; Jay et al., 2001). Thus, considering that the animals in the present study performed the subsurface swimming trials at their individual comfort, and the similarities with described underwater behaviour of wild walruses, measured DMR_{Swim} could be considered as a suitable approximation of the energetic swimming requirements for walruses when traveling to and from foraging areas or over long-distance migrations. However, the experimental procedures at facilities housing animals are not usually representative of the wild, and differences in underwater behaviours (e.g., social interactions, exploratory behaviours, etc.), or environmental conditions (e.g., water current, water temperature, etc.) experienced by free-ranging walruses could result in different energetic requirements during diving and swimming. This could explain why the observed $sDMR_{Swim}$ in the present study (see Table S2) is ~2/3 lower than the mass-specific FMR measured in two male adult Atlantic walruses, as that approximation accounted for behaviours in the wild (Acquarone et al., 2006). Despite this inherent limitation, these novel metabolic measurements on adult female walruses in water, could help improve energetic requirements estimations during these travelling subsurface dives, while helping interpret measurements of FMR associated with behavioural information.

6.5.3. Aerobic limitation estimated in the present study

The aerobic dive limit (ADL, min) was defined by Kooyman et al. (1980) as the longest dive duration that an animal can reach by exclusively using the available O_2 stores while avoiding the metabolic anaerobic pathway and the subsequent lactate accumulation in the blood. The estimated cADL in the present study (7.7 min) was longer than the dive duration for both underwater behaviours (stationary dives = 3.2 ± 0.1 min; subsurface swimming = 1.6 ± 0.2 min), and the duration of travelling and foraging dives observed in free-ranging male Pacific walruses that were suggested to occur within the ADL (median dive duration = 7.2 min, Jay et al., 2001). Therefore, the dives by females in the present study were well within the estimated cADL and likely supported by aerobic metabolism. In addition, the cADL of the walruses in this study was also longer than the average durations for most frequent dives (5-6 min) observed in adult male walruses foraging over continental shelf habitats at depths from 10 to 70 m (Acquarone et al., 2006; Gjertz et al., 2001; Wiig et al., 1993). However, walruses also take deeper and longer dives, exceeding

100 m depth (Acquarone et al., 2006) with a maximum reported dive depth of 234 m (Born et al., 2005) and a dive duration of 24 min (Gjertz et al., 2001). While, reported cADL in the present study could be overestimated due to lower energetic demands derived in laboratory conditions, if hypometabolism is related to dive duration in the walrus as in other pinnipeds, cADL may be in fact longer during extended dives. Thus, further investigations of the metabolic requirements for dives of different duration in this species would help clarify whether these animals are able to achieve the reported prolonged dive durations (>10 min, Acquarone et al., 2006; Gjertz et al., 2001; Wiig et al., 1993) while avoiding the blood lactate accumulation.

6.6. Conclusions

Walruses are currently facing changes to their environment that have altered the availability of their sea ice habitat, which have resulted in changes in their travelling and foraging behaviour, and geographical distribution (Jay et al., 2012; Udevitz et al., 2017). This has led to an increased concern about the future of the walrus populations, raising interest in studies aimed to obtain information about basal, reproductive, or diving and swimming energetic demands (Noren et al., 2012; Noren et al., 2014; Rosen, 2020; Udevitz et al., 2017). Studies measuring the energetic costs for different behaviours and life stages will help improve the accuracy of previous energetic models to predict the potential impacts of environmental change (Udevitz et al., 2017). The present study reports the first direct measurements of metabolic rates in female adult Pacific walruses while floating in water and during short and shallow stationary dives and subsurface swimming. The results are consistent with previous work reporting a low energetic cost of diving in marine mammals and confirms that the walruses are able to reduce their metabolic rates during both stationary dives and subsurface swimming. These metabolic estimates would help quantify energetic consequences of reported increased in-water behaviours in the walrus (Jay et al., 2012). In addition, as a benthic feeding marine mammal, walruses spend 70-93% of their time in water of which much of this time is spent diving to the ocean floor to feed on benthic invertebrates (Jay et al., 2017; Udevitz et al., 2009). Therefore, while the data reported in this study would help improve bioenergetic models, further investigations measuring the metabolic rate for different types of dives (e.g., longer durations or higher swimming requirements), and/or environmental parameters (e.g., water temperature or wind and water current), will help quantify the effect of these parameters on metabolic rates and further improve these

models. Similarly, measurements of the metabolic demands while resting on land would add relevant information that will also improve theoretical models aimed at forecasting the conservation scenario of this species.

6.7. Acknowledgements

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6.8. Supplementary information

6.8.1. Description for cADL calculation

The cADL was estimated by applying the methodology described in Kooyman (1989) and using specific estimates of tissues and oxygen-binding proteins proportions for walruses when available. The following assumptions were used to estimate the tissues (blood, muscle, and lungs) O_2 storage capacity and utilization during the dive. The total blood volume (BV, l) was calculated using the mass-specific estimation of 0.106 l blood kg^{-1} (Lenfant et al., 1970). The estimated proportions of arterial and venous blood were considered as 33% and 67% respectively (Lenfant et al., 1970). The initial and final arterial haemoglobin (Hb) saturation were assumed to be 95% and 20%, respectively. The initial venous O_2 content was assumed to be 5 vol% less than the initial arterial O_2 content, with a final venous O_2 content of zero (Ponganis, 2011). The Hb content of adult female walruses has been reported to be 16.8 g Hb 100 ml^{-1} of blood (Wołk and Kosygin, 1979), and the oxygen-binding capacity is 1.34 ml O_2 g^{-1} Hb (Kooyman, 1989). The final equations utilized for the calculation of total volume of blood O_2 (l) were:

1) Arterial O_2 = (BV · M_b · 0.33) · (0.95-0.20 saturation) · ([Hb] · 0.00134)

2) Venous O_2 = (BV · M_b · 0.67) · (initial arterial O_2 content-5vol%)

The total muscle O_2 storage capacity was estimated by calculating the total muscular myoglobin (Mg) content. The total muscle mass (kg) was calculated as 0.2410 $M_b^{1.084}$, an equation derived from excised tissues of Atlantic walruses (Knutsen and Born, 1994). The specific Mg content for the longissimus dorsi muscle in adult walruses has been estimated to be 3.8 g Mg 100 g^{-1} of wet muscle mass (Noren et al., 2015) and was assumed equal for all muscle groups. However, this approach can potentially overestimate the overall muscle O_2 storage capacity as lower levels of Mg have been reported for non-swimming muscles in pinnipeds (Kanatous et al., 1999). The Mg has been reported to possess the same oxygen-binding capacity than that reported for Hg (Kooyman, 1989). Thus, the equation used for the calculation of total volume of muscle O_2 (l) was:

3) Muscle O_2 = Total muscle mass · ([Mg] · 0.00134)

The total lung O_2 storage capacity was computed by calculating the estimated total lung capacity (TLC_{est}, l) using previous equation for marine mammals (TLC_{est} = 0.135 $M_b^{0.92}$, Fahlman et al., 2011; Kooyman, 1973). The diving lung volume was assumed to be 50% of TLC_{est} for pinnipeds with a 15% of available O_2 concentration in the lungs to be extracted during the dive (Ponganis, 2011). The final equation to yield the calculation of total volume of lung O_2 (l) was:

4) Lung O_2 = M_b · TLC_{est} · 0.5 · 0.15

All calculations were made using the overall average M_b for the three participating female walruses during the experiments (835 kg). The total body O_2 storage capacity (37.62 l O_2) was computed by summing the estimated O_2 storage capacity for each tissue. The cADL was computed by dividing the resulted total body O_2 storage capacity by the measured average DMR_{Swim} (4.91 l O_2 min^{-1}).

Table S4. Body mass and respiratory variables for each metabolic experiment in Pacific walruses.

Experiment	M_b (kg)	f_R (breaths min^{-1})	$\dot{V}CO_2$ (l CO_2 min^{-1})	RER
Floating at the water surface	844 ± 124 (697-1030)	5.8 ± 2.3 (3.1-9.6)	4.47 ± 0.94 (2.93-5.92)	0.97 ± 0.05 (0.90-1.04)
Stationary dive	827 ± 108 (697-967)	6.0 ± 1.9 (3.7-9.9)	3.58 ± 0.62 (2.84-4.47)	0.94 ± 0.07 (0.81-1.06)
Subsurface swimming	834 ± 113 (688-972)	6.6 ± 1.9 (3.6-9.7)	4.58 ± 0.58 (3.67-5.49)	0.94 ± 0.06 (0.85-1.05)

For the three adult female Pacific walruses (*O. rosmarus divergens*) participating in metabolic measurements while floating at the water surface, and after performing stationary dives and horizontal subsurface swimming (n = 15 for each respirometry experiment), average (± s.d.) and ranges of: body mass (M_b), respiratory frequency (f_R), CO_2 production rate ($\dot{V}CO_2$) and respiratory exchange ratio (RER).

Table S3. Summary of metabolic measurements in Pacific walruses converted to daily energetic requirements.

ANIMAL ID	$MR_{Surface}$ (MJ day^{-1})	$sMR_{Surface}$ (kJ kg^{-1} day^{-1})	$DMR_{Stationary}$ (MJ day^{-1})	$sDMR_{Stationary}$ (kJ kg^{-1} day^{-1})	DMR_{Swim} (MJ day^{-1})	$sDMR_{Swim}$ (kJ kg^{-1} day^{-1})
26005388	109.3 ± 13.4	154.0 ± 17.9	105.6 ± 9.4	149.9 ± 12.2	117.9 ± 8.0	167.9 ± 9.4
26005389	165.5 ± 20.2	166.6 ± 24.8	132.5 ± 6.9	138.1 ± 6.7	165.5 ± 5.9	171.0 ± 5.8
26005390	141.5 ± 26.4	171.3 ± 30.7	102.2 ± 13.4	124.8 ± 16.3	154.0 ± 3.7	185.2 ± 4.6
Grand Mean	138.8 ± 30.6 (95.0-193.7)	164.0 ± 24.4 (126.3-203.9)	113.4 ± 16.9 (87.0-139.8)	137.6 ± 15.6 (105.9-163.0)	145.8 ± 21.7 (106.5-172.7)	174.7 ± 10.1 (154.8-190.1)

For each participating female (Animal ID) Pacific walrus (*O. rosmarus divergens*, n = 3) and behaviour, average (± s.d) of measured metabolic rate (floating at the water surface: $MR_{Surface}$; stationary dives: $DMR_{Stationary}$; subsurface swimming: DMR_{Swim}) and mass-specific metabolic rate (floating at the water surface: $sMR_{Surface}$; stationary dives: $sDMR_{Stationary}$; subsurface swimming: $sDMR_{Swim}$). N = 5 for all experiments with each individual. Overall average (± s.d) and ranges are also reported for each behaviour.

6.9. List of symbols and abbreviations

- $\%kJ_{tot}$ = percentage of energy intake in relation to the total daily intake for the days of the experiments
- ADL = Aerobic dive limit, or maximum diving time exclusively using the available O_2 stores
- BMR = Basal metabolic rate
- BMR_{est} = Predicted basal metabolic rate
- BV = Blood volume
- cADL = Aerobic dive limit calculated from estimated body oxygen stores and diving metabolic rate during subsurface swimming dives
- DMR = Diving metabolic rate
- $DMR_{Stationary}$ = Metabolic rate measured during stationary dives
- DMR_{Swim} = Metabolic rate measured during subsurface swimming
- FMR = Field metabolic rate
- f_R = Respiratory frequency
- Hg = Haemoglobin
- kJ_{est} = Estimated energy intake
- M_b = Body mass
- Mg = Myoglobin
- $MR_{Surface}$ = Metabolic rate measured while floating at the water surface
- RER = Respiratory exchange ratio
- RMR = Metabolic rate measured at rest
- $sDMR_{Stationary}$ = Mass-specific metabolic rate measured during stationary dives
- $sDMR_{Swim}$ = Mass-specific metabolic rate measured during subsurface swimming
- $sMR_{Surface}$ = Mass-specific metabolic rate measured while floating at the water surface
- STPD = Standard temperature and pressure dry conditions
- TLC_{est} = Estimated total lung capacity
- $\dot{V}CO_2$ = Carbon dioxide production rate
- $\dot{V}O_2$ = Oxygen consumption rate

6.10. References

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7. General conclusions

Bottlenose dolphin, *Tursiops truncatus* (Montagu, 1821)
Red Sea, 2021
Photo credits: Alfredo Bernabeu Adrían

7. General conclusions

The present investigation was devoted to increase the current knowledge of respiratory physiology and energetic requirements in the Pacific walrus. In addition, the present study further aimed to evaluate the use of spirometry as a novel non-invasive tool to assess respiratory health in the bottlenose dolphin. The investigations presented in this thesis have inherent value within the field of marine mammal physiology, as these will add novel specific information for the Pacific walrus. This basic information could help improve theoretical models aimed to predict the physiological and energetic capacities and limitations during diving in this species, which, in turn, could help interpret the behavioural decisions undertaken by walruses in a changing environment. Similarly, the experimental evidence indicating the potential value of spirometry as a diagnostic tool for evaluation of respiratory disease in the bottlenose dolphin, provides a non-invasive method to help assist wild animals. Therefore, the contribution of the present thesis could also have relevant consequences for improving future wildlife management efforts and determining conservation priorities. The implications of these results have been discussed in detail in the corresponding Chapters I-III, and the present section highlights the main conclusions derived from each investigation.

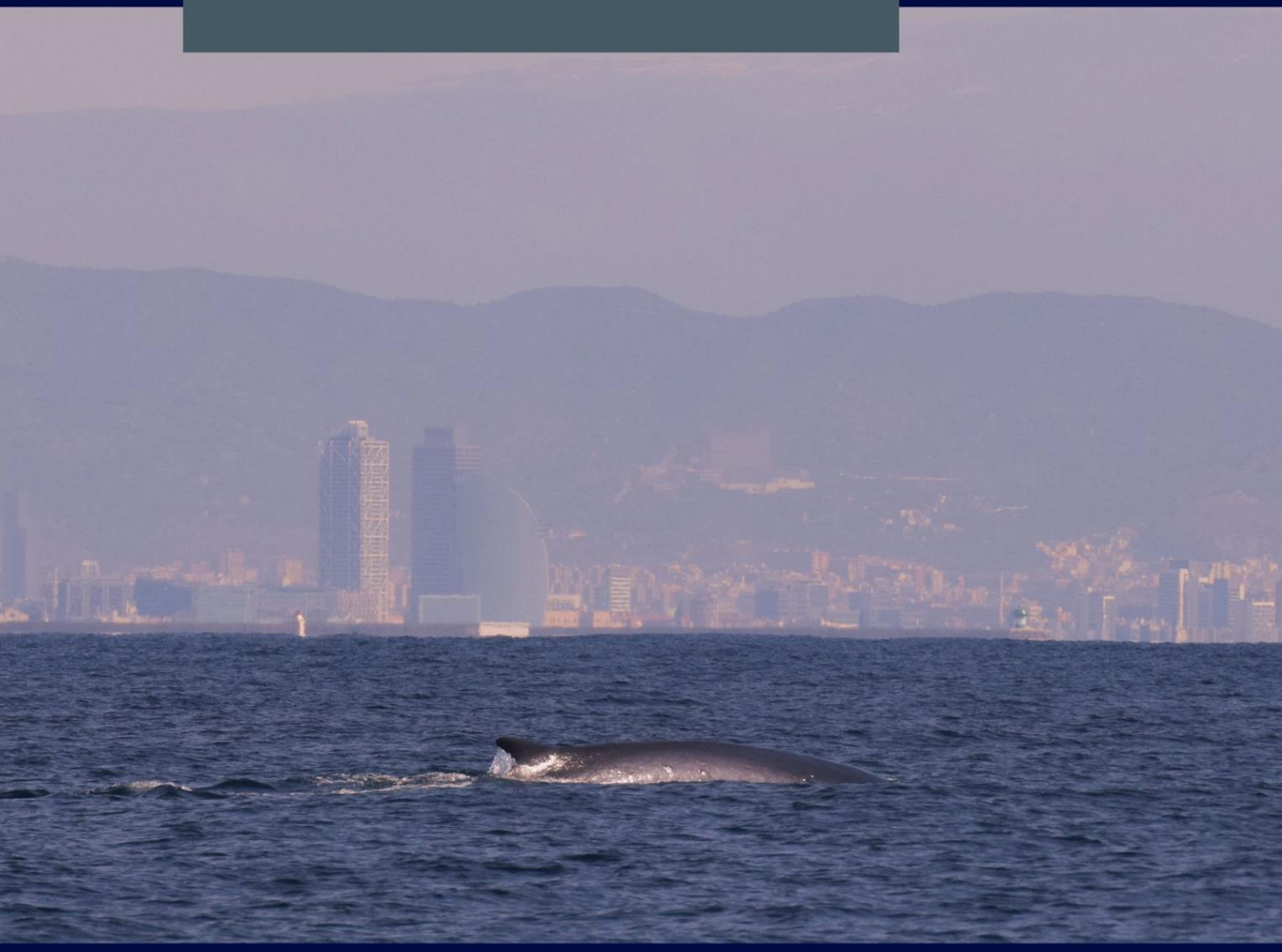
The results presented in Chapter I provide novel data about lung function and respiratory capacity in the Pacific walrus. These results are similar to those previously reported in pinnipeds and cetaceans that show an enhanced ventilatory capacity (e.g., higher tidal volume and lung compliance, and high respiratory flows maintained over the entire respiratory manoeuvre) as compared with terrestrial mammals. The assessment of lung function in different body positions on land and in water indicate flow limitations while lying on land. Additionally, the results suggest that the hydrostatic pressure may assist the elastic recoil of the lungs during the expiratory manoeuvre while in water, helping increase the expiratory flow as compared to when on land, which agrees with results reported in California sea lions. Therefore, the results presented in this chapter suggest that studies in semi-aquatic species should evaluate lung function both on land and in water. In addition, further investigations evaluating respiratory function and mechanics from individuals of different age and sex, would help understand the development of the respiratory capacity in this species.

The results presented in Chapter II suggest that lung function testing, or spirometry, can be used as a valuable diagnostic tool to detect respiratory disease and evaluate treatment efficacy in dolphins trained to exhale maximally while in water. These results also indicate that spirometry could provide additional information about the type (obstructive or restrictive) and location of the respiratory disease. In addition, the results obtained in dolphins temporarily beached suggest that this non-invasive tool could help define the respiratory health status of stranded individuals while breathing spontaneously. Additional studies in healthy individuals and animals with lung disease, could provide reference baseline values and help relate different types of respiratory disease with the developed lung function indices and flow-volume relationship. Thus, future studies could provide a non-invasive method that would be helpful to evaluate respiratory health and treatment efficacy in dolphins under human care, and a useful tool for in situ conservation purposes.

The results presented in Chapter III provide novel measurements of energetic requirements in adult female Pacific walruses while floating at the water surface, and during short and shallow stationary dives and subsurface swimming. These results are in agreement with previous work in other marine mammal species that reported an increased metabolic rate during inactive or resting periods as compared with terrestrial mammals of similar size. In addition, the results are similar to previous studies that showed a decreased metabolic rate during diving when compared with periods at the surface. This confirms that the walrus possesses physiological adaptations for an efficient use of O_2 during breath-hold. While this investigation did not evaluate whether the energetic requirements decrease with dive duration, as previously reported in other marine mammals, further investigations measuring the metabolic investment for longer dives would help determine the metabolic capacities in this species. In addition, studies evaluating the energetic requirements on land or under different environmental conditions in water, would help improve our understanding about the metabolic cost of resting and diving behaviour in the wild. The data presented in this chapter could help improve bioenergetic models aimed at quantifying the consequences of environmental change on observed changes in walrus behaviour. This, together with obtained results in Chapter I, could help define the physiological scope of this species and the survival limitations for individual animals and walrus populations.

In summary, the present thesis shows the value of physiological studies where the subjects are trained to perform specific experimental procedures while deciding their participation during data collection. Desensitization to the equipment likely reduces the potential stress associated with exposure to novel environments or procedures. Controlled experiments also help account for confounding variables that are difficult, or impossible to control in the wild (e.g., health status, body mass, age, sex, reproductive state, activity, etc.). In addition, cooperation of trained animals allows development of new equipment that can first be validated before being used on animals in the wild. Still, working with trained animals also has inherent limitations that should be considered (e.g., unpredictable voluntary compliance, the possible influence of the trainers on the behaviour, etc.). Facilities housing animals do not provide environmental parameters representative of wild conditions (e.g., water current, water temperature, pressure, etc.), and result in behavioural limitations (e.g., dive depth, swimming velocity, etc.). This has raised concerns about the physiological capacities of animals housed under professional care. Nevertheless, although some researchers consider that physiological results from these animals may have limited value, cooperation provides the opportunity to obtain data while prioritizing animal welfare, whereas similar studies would be logistically and ethically challenging in wild megafauna. Thus, studies on trained animals should be designed and interpreted considering their inherent limitations, where proper desensitization to the procedures and evaluation of collected data are crucial. In the present thesis, the extended desensitization and training prior to data collection, and the consistency of the behaviour during the experimental periods provide confidence of obtained results. Nevertheless, unravelling the relationship between different environmental and physiological factors affecting physiology and behaviour would require a combination of information gained from both, laboratory and field studies, to allow for an improved understanding of the relationship between physiology, environment and behaviour.

8. Resumen (castellano)



Fin whale, *Balaenoptera physalus* (Linnaeus, 1758)
Barcelona, Mediterranean Sea, 2021
Photo credits: Clara Agusti Pujol

8. Resumen (castellano)

8.1. Introducción

Las sucesivas incursiones de los primeros ancestros evolutivos de los mamíferos marinos en el medio marino, junto a la acción de los mecanismos evolutivos, favorecieron el desarrollo progresivo de adaptaciones anatómicas y fisiológicas que permitieron la colonización del medio acuático. Una de las mayores restricciones que este grupo de mamíferos superó fue la gran separación entre la obtención de los recursos alimenticios de origen marino y el O_2 atmosférico. Por lo tanto, la adquisición de adaptaciones específicas que permitieran un uso eficaz del O_2 durante la búsqueda de alimento fue vital para sobrevivir en el medio marino. La capacidad del buceo en mamíferos marinos ha sido fuente de interés desde las investigaciones más tempranas, y suscitó preguntas al respecto de los mecanismos fisiológicos que permitirían a estos animales alcanzar elevadas profundidades durante largos periodos de tiempo. Laurence Irving y Per Scholander lideraron las primeras investigaciones que estudiaron la relación entre la anatomía y la función cardiorrespiratoria en mamíferos marinos, y sus capacidades de buceo. Los resultados obtenidos sugirieron que estos animales tenían adaptaciones fisiológicas que les permitirían una mayor capacidad de almacenamiento de O_2 que los mamíferos terrestres, y una reducción de su utilización durante el buceo. Estas investigaciones iniciales generaron la base para el desarrollo de los estudios posteriores en el campo de la fisiología del buceo, los cuales, han destacado la importancia de la función pulmonar y del uso del O_2 durante el buceo en este grupo de mamíferos para permitir una exitosa explotación del medio marino.

En el campo de la fisiología respiratoria, los primeros estudios anatómicos de Scholander mostraron que algunas especies de focas y cetáceos poseían pulmones y cajas torácicas muy flexibles, así como vías respiratorias superiores reforzadas. Scholander sugirió que estas características anatómicas podrían permitir que el aire contenido en los pulmones se desplazase a las vías respiratorias superiores menos compresibles con el aumento de la presión hidrostática en profundidad. De esta forma, se produciría un colapso alveolar en profundidad que, en última instancia, cesaría el intercambio gaseoso de los pulmones, disminuyendo la acumulación de N_2 y el riesgo de padecer enfermedad descompresiva. Sin embargo, este refuerzo de las vías respiratorias superiores en mamíferos marinos también fue sugerido como una posible adaptación que permitiría la

generación de flujos respiratorios ($\dot{V}$) elevados. Estudios posteriores sobre anatomía y función pulmonar en animales vivos, demostraron que el refuerzo de las vías respiratorias, la elevada flexibilidad de los pulmones y la caja torácica, y la generación de elevados $\dot{V}$ durante respiraciones de corta duración, era extensible a otras especies de mamíferos marinos. Algunos de estos estudios mostraron que los elevados $\dot{V}$ son mantenidos casi constantes durante la totalidad de la maniobra respiratoria. Además, algunas especies de cetáceos han demostrado tener la capacidad de intercambiar hasta el 90% de su capacidad total pulmonar (TLC) en una única respiración. Sin embargo, el volumen corriente (V_T) durante la mayor parte de las maniobras respiratorias, parece encontrarse por debajo de estos valores extremos de intercambio. Aun así, los mamíferos marinos tienen un V_T mayor y una frecuencia respiratoria (f_R) menor, que las estimaciones esperadas para mamíferos terrestres de tamaño similar. No obstante, la información obtenida de estos estudios no sólo ha mostrado las diferencias de la función respiratoria en mamíferos marinos con respecto a sus congéneres terrestres, sino que también ha puesto de manifiesto una gran variabilidad anatómica y funcional entre las distintas especies de mamíferos marinos. A pesar de esta variabilidad, la evidencia científica indica que, en general, la arquitectura anatómica del sistema respiratorio de los mamíferos marinos proporciona una capacidad ventilatoria muy eficaz, además de evitar problemas asociados con la presión. Esta gran capacidad de intercambio les permitiría disminuir los intervalos de recuperación en superficie durante los que se restauran los valores de O_2 y se elimina el CO_2 producido durante el buceo. Por lo tanto, el sistema respiratorio cumple un papel fundamental en el rápido intercambio de gases en superficie, así como en la determinación de la cantidad de O_2 que podrá ser utilizado durante el buceo.

Después de regresar a la superficie para eliminar el CO_2 acumulado y renovar el O_2 necesario para la siguiente inmersión, los mamíferos marinos cuentan con una cantidad limitada de O_2 para lograr el objetivo del buceo. A principios de los años 40, Irving y Scholander desarrollaron una serie de experimentos con mamíferos marinos forzados a retener su respiración, para comprender los mecanismos fisiológicos que permitían los largos periodos de apnea. Estos estudios mostraron un descenso en el ritmo cardiaco (o bradicardia), una reducción de la tasa de consumo de O_2 arterial, y un aumento de la concentración de lactato en sangre. Estos resultados fueron relacionados con una vasoconstricción de los órganos periféricos y una perfusión selectiva a aquellos órganos con requerimientos constantes de O_2 (cerebro, pulmones y corazón). Los órganos aislados

del aporte de O_2 producirían energía a través de la vía anaerobia, con el consecuente incremento de lactato en sangre. El descenso del trabajo cardiaco y la reducción del uso del O_2 por parte de los tejidos menos perfundidos, ayudaría a explicar la reducción observada en el consumo de O_2 durante el buceo. Estudios posteriores confirmaron esta respuesta cardiovascular durante buceos libres en otras especies. Además, otros estudios demostraron que la intensidad de esta respuesta podía ser modulada en función de los requerimientos de la inmersión. Posteriormente, en los años 80, el desarrollo de nuevas tecnologías de seguimiento permitió recabar información sobre la duración y profundidad del comportamiento del buceo en diferentes especies, observándose que los buceos rutinarios de los mamíferos marinos solían estar alejados de los buceos más extremos. Estas observaciones sugirieron que los mamíferos marinos realizarían la mayor parte de sus inmersiones utilizando la vía aeróbica, evitando la vía anaeróbica y el consecuente aumento del lactato en sangre. Esta estrategia, permitiría a los mamíferos marinos reducir los intervalos de recuperación en superficie requeridos para eliminar los subproductos derivados del metabolismo anaerobio. El concepto de límite aérobico del buceo (ADL) fue acuñado para definir el tiempo máximo durante el cual se podría realizar una inmersión utilizando las reservas de O_2 sin la acumulación de lactato en sangre. Dada la dificultad de medir las concentraciones de lactato en sangre, el ADL ha sido frecuentemente estimado (cADL) a través del cálculo del O_2 total disponible durante la inmersión, dividido por el consumo de O_2 (o tasa metabólica) durante el buceo. Por ello, estudios posteriores tuvieron como objetivo medir la tasa metabólica del buceo (DMR) para comparar el cADL con observaciones comportamentales, y comprender mejor las condiciones metabólicas durante el buceo. Estos estudios han mostrado que la DMR es frecuentemente menor o similar a la tasa metabólica medida en periodos de descanso en superficie, y que la DMR se reduce proporcionalmente con respecto a la duración del buceo. Además, algunos de estos estudios han indicado que el grado de reducción de la DMR podría ser muy variable entre las diferentes especies, lo cual, ha sido relacionado con las diversas estrategias de forrajeo. Por otra parte, las comparaciones realizadas entre el cADL y los datos obtenidos sobre comportamientos de buceo, han indicado que algunas especies de comportamiento bentónico podrían exceder regularmente el cADL. Por lo tanto, la evidencia experimental ha demostrado que los mamíferos marinos poseen mecanismos fisiológicos para extender los tiempos de inmersión aeróbicos, siendo un componente importante el uso eficiente de O_2. Sin embargo, el alcance interespecífico de

estos mecanismos fisiológicos, así como su relación con determinadas circunstancias comportamentales todavía es poco conocido.

Los estudios sobre anatomía respiratoria, función pulmonar, y requerimientos energéticos en mamíferos marinos han proporcionado un marco teórico general para comprender sus capacidades respiratorias y de buceo. Esta información ha sido fundamental para realizar modelos matemáticos que permiten definir las de este comportamiento. Sin embargo, estos estudios también han destacado diferencias interespecíficas significativas que impiden realizar extrapolaciones sobre ciertos parámetros básicos en aquellas especies menos estudiadas. La supervivencia de los mamíferos marinos depende de sus capacidades de buceo, ya que éstas determinan su eficacia durante el forrajeo. Sin embargo, el creciente impacto de la actividad humana está resultando en alteraciones en el medio marino (p.ej., cambio climático, sobrepesca, contaminación etc.) que podrían forzar el comportamiento de los mamíferos marinos por encima de sus capacidades fisiológicas. Por ejemplo, estudios en cetáceos varados han mostrado lesiones severas asociadas a la aparición de embolia gaseosa tras la exposición a ruido submarino de origen antrópico. Por otra parte, la enfermedad respiratoria es considerada una de las mayores causas de mortalidad en mamíferos marinos, y las alteraciones en el medio marino podrían disminuir la capacidad de respuesta del sistema inmune de estas especies, incrementando la susceptibilidad ante determinados patógenos. Además, los cambios en la disponibilidad de los principales recursos alimenticios de los mamíferos marinos podrían resultar en la realización de buceos por encima de sus capacidades fisiológicas. Las proyecciones futuras sobre el creciente impacto en el medio marino reflejan la necesidad de mejorar nuestro conocimiento sobre las capacidades y limitaciones fisiológicas de los mamíferos marinos para ayudar en su conservación. Por lo tanto, estudios que proporcionen parámetros básicos de función pulmonar y de requerimientos energéticos en diferentes especies de mamíferos marinos, podrán ayudar a mejorar modelos teóricos que tengan como objetivo predecir sus limitaciones fisiológicas, o comprender el impacto de los cambios ambientales en su comportamiento. De la misma forma, estas bases de datos pueden ayudar a comprender las consecuencias de la enfermedad respiratoria en la función pulmonar, y proporcionar la oportunidad de desarrollar nuevas metodologías para evaluar la salud respiratoria en estos animales.

8.2. Objetivos

El objetivo principal de la presente tesis fue mejorar el conocimiento científico sobre fisiología básica en mamíferos marinos a través del estudio de animales entrenados. La realización del presente trabajo de investigación fue posible gracias a la colaboración entre la Universitat de València, la Fundación Oceanogràfic de la Comunitat Valenciana, e instalaciones que albergan mamíferos marinos bajo cuidado profesional. El objetivo principal fue desarrollado a través de objetivos específicos que explorasen cuestiones de las que se tuviera escaso conocimiento previo, y que permitieran un mayor conocimiento de las capacidades y limitaciones de los mamíferos marinos, con el fin de mejorar las herramientas actuales de conservación. Dichos objetivos se dividieron en tres capítulos independientes:

Capítulo I: Función pulmonar en la morsa del Pacífico: El objetivo de este capítulo fue medir parámetros básicos de función pulmonar en individuos adultos de morsa del Pacífico. Además, se comprobó el posible efecto de la presión sobre la caja torácica en la función pulmonar a través de la recolección de datos en diferentes posiciones corporales en tierra y en el agua.

Capítulo II: Espirometría como método diagnóstico en el delfín mular: Este capítulo tuvo como objetivo la evaluación del uso de la espirometría como método no invasivo para el diagnóstico de la enfermedad pulmonar en el delfín mular.

Capítulo III: Tasas metabólicas en la morsa del Pacífico: Este capítulo se centró en la determinación de los requerimientos energéticos de la natación superficial y de apneas inactivas en individuos adultos de morsa del Pacífico. Además, estos datos fueron comparados con los obtenidos durante periodos flotando en la superficie del agua.

8.3. Capítulo I: Función pulmonar en la morsa del Pacífico

Introducción

Los mamíferos marinos deben regresar a la superficie para renovar el O_2 utilizado y eliminar el CO_2 acumulado durante el buceo. Por ello, la eficacia del intercambio del aire contenido en los pulmones es importante para una rápida renovación del O_2 antes de reanudar la actividad subacuática. Las adaptaciones anatómicas y funcionales del sistema respiratorio en mamíferos marinos son cruciales para el intercambio de gases en superficie. Desde las primeras investigaciones lideradas por Irving y Scholander, los

estudios sobre anatomía y función pulmonar han permitido generar una idea general sobre las adaptaciones del sistema respiratorio en estos animales, mostrando las diferencias con respecto a sus congéneres terrestres, y destacando su relevancia en la determinación de las limitaciones del buceo. Sin embargo, estos estudios también han revelado una elevada variabilidad interespecífica que impediría la generalización de ciertos aspectos básicos de función pulmonar para aquellas especies de las que se tiene poca información. Los crecientes impactos ambientales en el océano podrían resultar en una modificación del comportamiento del buceo en algunas especies. Este posible escenario futuro aumenta la necesidad de recopilar información para mejorar nuestra comprensión sobre las limitaciones fisiológicas de las diferentes especies. Por ello, el principal objetivo del presente capítulo de tesis fue recoger datos sobre parámetros básicos de función pulmonar en una especie de la que se había obtenido escasa información previa: la morsa del Pacífico. Además, teniendo en cuenta que un estudio reciente en leones marinos de California había mostrado diferencias significativas en la función pulmonar dependiendo de la posición corporal, el presente estudio también tuvo como objetivo la evaluación de estas posibles diferencias en las morsas.

Material y métodos

Tres hembras adultas de morsa del Pacífico [*Odobenus rosmarus divergens* (Illiger, 1815)] alojadas en el Oceanogràfic de València, fueron entrenadas para participar en la recolección de datos de función pulmonar mediante el uso de la espirometría. Las sesiones de espirometría tuvieron lugar entre febrero de 2015 y julio de 2018, y se realizaron en tres posiciones corporales diferentes: 1) tumbadas en decúbito esternal, 2) sentadas en posición erguida mantenidas con las aletas pectorales, y 3) flotando en vertical en la superficie del agua. Las tres morsas realizaron respiraciones normales (o espontáneas) en las tres posiciones, y dos de las morsas fueron entrenadas para realizar esfuerzos respiratorios máximos mientras se encontraban sentadas. Además, una de las morsas fue entrenada para la inserción de una sonda conectada con un balón esofágico, con el fin de estimar la distensibilidad pulmonar (C_L) durante respiraciones espontáneas (o C_L dinámica). Los animales no presentaron enfermedad pulmonar durante el periodo experimental, y su masa corporal (M_b) se midió la misma semana en la que se realizaron las sesiones de espirometría.

Las espirometrías fueron realizadas gracias a la utilización de un neumotacómetro (o espirómetro) de Fleisch especialmente diseñado para la especie objeto de estudio. Una

matriz de baja resistencia al flujo laminar fue colocada dentro del espirómetro para crear una diferencia de presión a ambos lados de la matriz durante la recolección de datos respiratorios. Esta diferencia de presión fue medida para su conversión a unidades de $\dot{V}$ mediante el uso de una jeringa de calibración de volumen conocido. Para cada maniobra respiratoria, el $\dot{V}$ obtenido durante la espiración ($\dot{V}_{exp}$) e inspiración ($\dot{V}_{insp}$) fueron integrados para calcular el V_T espiratorio (V_{Texp}) e inspiratorio (V_{Tinsp}) respectivamente. El V_T fue convertido a condiciones estándar de temperatura, presión, y humedad (0%). Además, también se obtuvieron la duración de la espiración (T_{exp}) e inspiración (T_{insp}), y la duración total de la respiración (T_{tot}). La f_R fue calculada para cada sesión de espirometría durante la toma de datos de respiraciones espontáneas. La C_L dinámica se calculó como el V_{Tinsp} dividido por la diferencia de presión entre la apertura de las vías respiratorias y la presión esofágica cuando el $\dot{V}$ es igual a cero. Dado que la C_L varía en función del tamaño pulmonar, se obtuvo la C_L dinámica específica o normalizada (sC_L) dividiendo la C_L dinámica obtenida por la estimación alométrica del volumen mínimo de aire que permanecería en el pulmón extirpado en mamíferos marinos. Únicamente aquellos muestreos en los que los animales estuvieron tranquilos y realizando respiraciones normales y completas fueron incluidos en el análisis.

Resultados y discusión

En total, 120 sesiones de espirometrías fueron realizadas con la colaboración de las tres hembras adultas de morsa del Pacífico, incluyendo sesiones de entrenamiento y de recolección de datos. Del total de muestreos, 45 fueron incluidos en el análisis (5 por posición corporal y animal). Además, 9 muestreos independientes con dos de las morsas incluyeron medidas de respiraciones máximas, y 3 muestreos independientes fueron utilizados para calcular la C_L dinámica.

El patrón respiratorio de las morsas fue similar al de otros pinnípedos, comenzando con una espiración, seguida de una inspiración y una pausa respiratoria. Con respecto a las posiciones corporales, el $\dot{V}$ fue menor cuando las moras se encontraban tumbadas ($\dot{V}_{exp}$ = 7,1 ± 1,2 l s^{-1}; $\dot{V}_{insp}$ = 5,5 ± 1,1 l s^{-1}) en comparación con cuando se encontraban en el agua ($\dot{V}_{exp}$ = 9,9 ± 1,4 l s^{-1}; $\dot{V}_{insp}$ = 7,2 ± 1,2 l s^{-1}). Sin embargo, la posición corporal no afectó a los valores obtenidos del V_T específico o normalizado por la M_b (sV_T, p. ej., espiración = 13,9 ± 5,2 ml kg^{-1}), T_{exp} (2,1 ± 0,7 s), T_{insp} (2,5 ± 0,9 s), o la f_R (7,6 ± 2,2 respiraciones min^{-1}). Estos resultados fueron similares a los resultados obtenidos en un estudio previo realizado con leones marinos de California, donde se

sugirió que el incremento de la presión hidrostática sobre la caja torácica podría ayudar a incrementar el $\dot{V}$. En mamíferos marinos la inspiración es activa, mientras que la espiración es pasiva y principalmente llevada a cabo por el retroceso elástico del pulmón. Por ello, la función pulmonar podría estar limitada por el incremento de presión sobre la caja torácica cuando los animales están tumbados. De hecho, el $\dot{V}$ obtenido mientras las morsas se encontraban tumbadas fue menor que el medido cuando estaban en el agua. Además, la presión hidrostática podría ayudar al retroceso elástico del pulmón durante la espiración, permitiendo alcanzar $\dot{V}_{exp}$ más elevados de forma pasiva cuando los animales están en el agua. Por último, aunque no se encontraron diferencias significativas en los parámetros medidos entre las dos posiciones fuera del agua, el posible efecto de la disminución de la presión sobre la caja torácica en la función pulmonar cuando los animales están sentados no debería ser descartado.

El V_T durante las maniobras espontáneas (p.ej., V_{Texp} = 11,5 ± 4,6 l) fue mayor que el estimado para un mamífero terrestre de tamaño similar, y aumentó con el incremento de la M_b con un exponente cercano a 1, coincidiendo con la tendencia previamente observada para otras especies de mamíferos marinos. El rango del V_T medido durante las maniobras respiratorias espontáneas varió entre el 3-43% de la TLC estimada (TLC_{est}), mientras que el valor máximo obtenido durante las maniobras respiratorias máximas (V_{Texp} = 31,9 l) alcanzó el 50% de la TLC_{est}. Estos valores coinciden con estudios previos en mamíferos marinos que indican que el V_T alcanzado durante la mayor parte de las respiraciones se encuentra normalmente entre un 20-40% de la TLC_{est}, y supera el 14% observado para mamíferos terrestres. La sC_L dinámica obtenida (0,32 ± 0,07 cmH_2O^{-1}) fue similar a datos previos en otras especies de mamíferos marinos, superando el rango de valores informados para mamíferos terrestres. Por lo tanto, las variables de función pulmonar medidas en el presente estudio coinciden con estudios previos en otras especies de mamíferos marinos que sugieren una capacidad de ventilación más eficiente en estos animales (p.ej., mayor V_T y sC_L), en comparación con los mamíferos terrestres.

Por otra parte, durante las maniobras respiratorias máximas, el $\dot{V}_{exp}$ normalizado por la TLC_{est} fue menor que para el león marino de California y algunas especies de cetáceos, pero mayor que el indicado para especies de fócidos. Además, las curvas de flujo-volumen mostraron que el $\dot{V}$ se mantiene casi constante a lo largo de toda la maniobra respiratoria, tanto para maniobras máximas como para espontáneas. La generación de elevados $\dot{V}$, que se mantienen constantes a lo largo de la maniobra

respiratoria, ha sido relacionada con el refuerzo de las vías aéreas superiores descrito en mamíferos marinos. Aunque un estudio más profundo ayudaría a relacionar las propiedades anatómicas del sistema respiratorio en morsas con la capacidad de generación de elevados $\dot{V}$, los resultados obtenidos indicarían que esta especie es capaz de generar y mantener $\dot{V}$ elevados y constantes durante el intercambio de aire pulmonar, tal y como ha sido descrito previamente en mamíferos marinos.

8.4. Capítulo II: Espirometría como método diagnóstico en el delfín mular

Introducción

La enfermedad pulmonar es uno de los problemas más extendidos entre los cetáceos, tanto en el medio natural, como para aquellos individuos alojados bajo cuidado profesional. Además, es común que los individuos enfermos no muestren signos clínicos de la enfermedad hasta encontrarse severamente afectados. Algunos de los métodos más habituales para diagnosticar enfermedad pulmonar proporcionan información sobre el origen de las infecciones respiratorias (p.ej., muestras de sangre o esputo, citología, etc.), mientras que otros métodos que evalúan la tipología de la enfermedad son generalmente invasivos y presentan complicaciones metodológicas a la hora de llevarlos a cabo en estas especies (p. ej., ecografías, radiografías, broncoscopia, etc.). Sin embargo, ninguno de estos métodos proporciona información sobre las consecuencias de la enfermedad respiratoria en la función pulmonar. La prueba de función pulmonar (PFT) o espirometría es el método estándar para evaluar la salud respiratoria en humanos. Estas pruebas se realizan midiendo el $\dot{V}$ durante una o varias maniobras respiratorias. Por ejemplo, es habitual pedir a los pacientes una inspiración completa hasta alcanzar la TLC, para luego realizar una espiración máxima. A partir de estas maniobras se obtienen una serie de índices numéricos y curvas de flujo-volumen, que permiten evaluar las posibles desviaciones en comparación con los valores y patrones normales esperados. Las PFT han sido previamente utilizadas para la evaluación de enfermedad respiratoria en mamíferos terrestres, por lo que esta técnica también podría ser una herramienta de diagnóstico en especies marinas donde es logísticamente complicado identificar y evaluar la progresión de la enfermedad pulmonar. Por ello, el objetivo del presente capítulo de tesis fue valorar la aplicación de las PFT para evaluar la salud respiratoria en el delfín mular.

Material y métodos

Tres individuos de delfín mular [*Tursiops truncatus*, (Montagu, 1821)] alojados en las instalaciones de Siegfried and Roy's Secret Garden (Las Vegas), fueron entrenados para participar en las PFT durante 6 periodos independientes de muestreo (entre abril de 2017 y abril de 2019). Los 3 individuos (1 hembra y 2 machos) participaron en las PFT flotando en el agua mientras realizaban maniobras respiratorias espontáneas y máximas. Además, los dos machos participaron en PFT puntuales durante varamientos entrenados y temporales realizados con fines veterinarios, mientras realizaban maniobras respiratorias espontáneas. Debido a las características del entrenamiento, de las maniobras respiratorias máximas, sólo se consideró como máxima la fase espiratoria. Las PFT se llevaron a cabo gracias a la utilización de un neumotacómetro de Fleisch especialmente diseñado para la especie objeto de estudio. La metodología para la obtención de los parámetros básicos de función pulmonar (V_T, $\dot{V}$, y T_{tot}) fue idéntica a la descrita en el Capítulo I. La salud respiratoria de cada individuo fue paralelamente evaluada a través de marcadores clínicos de muestras de sangre y esputo respiratorio (± 14 días desde la PFT), y radiografías torácicas (generalmente ± 30 días desde la PFT). Los animales fueron entrenados para participar en la adquisición de radiografías en diferentes posiciones corporales fuera del agua para permitir una evaluación completa de los pulmones.

Para evaluar el estado de salud pulmonar mediante las PFT, se obtuvieron las curvas de flujo-volumen y se definieron dos índices numéricos para la fase espiratoria en base a la metodología utilizada en medicina humana. El primero de estos índices se obtuvo utilizando el flujo espiratorio máximo (PEF), pero ajustado para la función pulmonar en delfines: el porcentaje de la capacidad vital (VC) espirada al alcanzar el PEF ($\%CV_{PEF}$). El segundo índice fue el flujo espiratorio medido entre el 25% y el 75% de la maniobra de espiración máxima o VC ($FEF_{25-75\%}$). De las PFT en el agua, se utilizaron las tres maniobras de mayor esfuerzo respiratorio para realizar las curvas de flujo-volumen y calcular el $FEF_{25-75\%}$ y $\%CV_{PEF}$. De las PFT durante los varamientos, se realizaron las curvas de flujo-volumen de todas las respiraciones espontáneas, y las 3 maniobras más similares fueron utilizadas para calcular el $FEF_{25-75\%}$ y $\%CV_{PEF}$.

Resultados y discusión

Los análisis clínicos y las radiografías realizadas durante el periodo experimental sugirieron tres casos clínicos diferentes en cuanto al estado de salud del sistema respiratorio de los tres delfines. Los animales fueron tratados para la enfermedad pulmonar, y el diagnóstico también incluyó la respuesta al tratamiento. La hembra (H1) no mostró signos de enfermedad activa a través de las analíticas, pero las radiografías mostraron anomalías persistentes e invariables, que sugirieron fibrosis pulmonar. El primero de los machos (M1) no presentó signos clínicos de enfermedad pulmonar a través de las analíticas, pero presentó un patrón anormal emergente a través de las radiografías (enero 2019), que fue interpretado como una posible neumonía que se resolvió a los tres meses de recibir tratamiento, por lo que M1 fue considerado asintomático. El último delfín macho (M2) mostró valores normales en las analíticas y radiografías, pero presentó anomalías clínicas a partir de enero de 2018 que fueron interpretadas como neumonía. Las radiografías posteriores de M2 mostraron neumonía persistente que empeoró en agosto de 2018, por lo que se modificó el tratamiento inicial. Posteriormente, los resultados radiográficos mostraron una mejoría a finales de 2018 y principios de 2019.

Los tres delfines participaron en un total de 18 sesiones experimentales durante las PFT en el agua. Para las maniobras respiratorias máximas, no hubo cambios en el V_T, $\dot{V}$, o T_{tot} durante el periodo experimental en ninguno de los delfines. Sin embargo, el sV_T y el $\dot{V}$ normalizado por la M_b ($s\dot{V}$) de F1 fueron menores (p.ej., sV_T espiratorio = 24 ± 3 ml kg^{-1}; $s\dot{V}$ espiratorio = 103 ± 12 ml s^{-1} kg^{-1}) en comparación con los rangos obtenidos para M1 y M2 (sV_T espiratorio = 56-60 ml kg^{-1}, $s\dot{V}$ espiratorio = 250-305 ml s^{-1} kg^{-1}). F1 no presentó cambios en ninguno de los índices pulmonares definidos, y la forma de las curvas de flujo-volumen fueron constantes, pero tanto la VC como el $FEF_{25-75\%}$ y el $\%VC_{PEF}$ fueron menores que los de M1 y M2. Además, estas curvas también mostraron que el PEF se alcanzaba al principio de la espiración, pero que el $\dot{V}_{exp}$ disminuía rápidamente durante el descenso del volumen pulmonar. La reducción en los parámetros básicos de función pulmonar (sV_T y $s\dot{V}$) en F1, sugiere una correspondencia apropiada con las radiografías obtenidas y el diagnóstico realizado en la hembra de delfín, dado que la fibrosis crónica es una enfermedad restrictiva que reduce la VC. Además, la limitación al $\dot{V}_{exp}$ conforme disminuye el volumen pulmonar, podría indicar una obstrucción en las vías aéreas inferiores. Por otra parte, M1 no presentó cambios significativos ni en los índices pulmonares ni en las curvas de flujo-volumen a lo largo del periodo experimental.

Además, las curvas de flujo-volumen indicaron un pulmón sano capaz de mantener elevados $\dot{V}$ a lo largo de toda la maniobra respiratoria. Estos resultados sugieren que, posiblemente, la neumonía detectada a través de las radiografías podría haber sido leve, de forma que no habría alterado la función pulmonar como para ser detectada en las PFT. Por último, M2 mostró un descenso del 27% en el $FEF_{25-75\%}$ y del 52% en el $\%VC_{PEF}$ cuando el animal fue diagnosticado con neumonía. Además, las curvas de flujo-volumen obtenidas después del diagnóstico, mostraron un patrón similar a F1, donde los $\dot{V}_{exp}$ disminuían después de alcanzar el PEF al principio de la espiración. Tanto los índices pulmonares como las curvas de flujo-volumen recuperaron, respectivamente, los valores y la forma inicial cuando el animal presentó mejoría en las radiografías. En el caso de M2, aunque la neumonía también es una enfermedad restrictiva, no se detectó una reducción en la VC. Sin embargo, los resultados obtenidos de los índices pulmonares y la limitación al $\dot{V}_{exp}$ en las curvas de flujo-volumen, podrían indicar una posible obstrucción pulmonar en las vías aéreas inferiores como en el caso de F1. Los resultados obtenidos de las PFT concuerdan con las pruebas diagnósticas realizadas en los tres delfines y, además, proporcionaron información adicional sobre posibles enfermedades concomitantes a la fibrosis y a la neumonía en F1 y M2 respectivamente, así como sobre su localización en las vías aéreas.

Los dos delfines macho realizaron un total de 4 PFT mientras realizaban respiraciones espontáneas durante dos varamientos temporales. Tanto para M1 como para M2, las curvas flujo-volumen cuando los animales estaban sanos mostraron un descenso en la VC y cierta limitación al $\dot{V}$, en comparación con las respiraciones espontáneas en el agua. Por otra parte, para M1 no hubo cambios significativos ni en los índices pulmonares ni en las curvas de flujo-volumen entre los dos muestreos durante el varamiento. Sin embargo, para M2, el $\%VC_{PEF}$ descendió un 49% y la curva de flujo-volumen mostró anomalías que indicaron limitaciones al $\dot{V}$ durante la PFT coincidente con el diagnóstico de neumonía. Durante el varamiento, los cetáceos sufren un incremento en la presión torácica debido a su propio peso, que podría explicar el descenso en la VC y la limitación al $\dot{V}$ durante respiraciones espontáneas en animales sanos y varados. En medicina humana, es habitual evaluar la posible obstrucción pulmonar a través de maniobras espontáneas en pacientes incapaces de realizar maniobras respiratorias máximas de forma voluntaria. Durante estas pruebas, se ha sugerido que la aplicación de una leve presión torácica aumentaría el esfuerzo respiratorio, pudiendo ser útil a la hora de mejorar la

evaluación. Por ello, la combinación del esfuerzo respiratorio durante el varamiento y la enfermedad pulmonar en M2, pudo resultar en una limitación respiratoria durante las maniobras espontáneas que permitió la detección de la enfermedad mediante las PFT durante el varamiento.

8.5. Capítulo III: Tasas metabólicas en la morsa del Pacífico

Introducción

La morsa del Pacífico habita una región del Ártico que está experimentando uno de los retrocesos más rápidos del hielo marino. El hielo marino es un hábitat crítico para las morsas, ya que funciona como un área de descanso, proporciona acceso rápido a las zonas de alimentación, y también es importante para el cuidado de las crías. La disminución de la extensión del hielo en el Ártico está incrementando la frecuencia del uso de zonas de descanso en la costa continental por parte de las morsas. Como consecuencia, algunos individuos recorren mayores distancias para buscar sus zonas habituales de alimentación, pasando más tiempo en el agua y menos tiempo descansando. Estos recorridos podrían incrementar el coste energético diario en esta especie, pudiendo tener consecuencias en la supervivencia a nivel individual. Comprender las implicaciones de los cambios de actividad en el balance energético de esta especie, requiere de información sobre la demanda energética de comportamientos concretos. Los dispositivos de seguimiento satelital permiten recoger información sobre la localización y la actividad de los individuos. Esta información, combinada con datos sobre el requerimiento energético de actividades específicas, podría ayudar a mejorar las predicciones de las respuestas comportamentales de las morsas a los potenciales cambios ambientales. Sin embargo, encontramos escasa información sobre los requerimientos energéticos para actividades concretas en morsas. Por ello, el objetivo del presente capítulo de tesis fue medir los requerimientos energéticos o tasas metabólicas de diferentes comportamientos en el agua en la morsa del Pacífico.

Material y métodos

Tres hembras adultas de morsa del Pacífico [*Odobenus rosmarus divergens* (Illiger, 1815)] alojadas en el Oceanogràfic de València, fueron entrenadas para participar en la recolección de datos de tasas metabólicas utilizando un sistema de respirometría de flujo abierto. Las sesiones experimentales tuvieron lugar entre agosto de 2018 y noviembre de 2019. La M_b de los animales se midió durante la misma semana de la sesión

experimental, y el estado de salud de los animales fue evaluado periódicamente. Los animales se encontraban en condiciones de ayuno al iniciar las sesiones experimentales.

La recolección de datos metabólicos se realizó utilizando una cúpula de Plexiglas posicionada en la superficie de una piscina. Esta cúpula poseía dos aperturas que permitían la entrada de aire atmosférico, y la conexión a un generador de flujo encargado de extraer el aire contenido en la cúpula a una tasa constante. Los animales fueron entrenados para introducirse en la cúpula y mantenerse dentro tranquilos mientras respiraban normalmente. Los cambios provocados por los animales en el contenido del aire atmosférico entrante en el sistema, fueron medidos con un analizador de O_2 y CO_2 a través de una submuestra del aire extraído por el generador de flujo. A partir de estos datos, se utilizó la tasa de consumo de O_2 para estimar la tasa metabólica durante tres actividades diferentes en el agua: 1) mientras los animales flotaban en la superficie durante al menos 5 minutos ($MR_{Surface}$), y durante 2) buceos estacionarios o inactivos de al menos 3 minutos de duración ($DMR_{Station}$), o 3) durante ~90 metros de natación horizontal subsuperficial (DMR_{Swim}). Al terminar la actividad subacuática, las morsas fueron entrenadas para introducirse inmediatamente dentro de la cúpula para medir el consumo de O_2 y la producción de CO_2 durante la recuperación del buceo. La terminación de la recuperación del buceo fue determinada como el momento en el que el consumo de O_2 y la producción de CO_2 recuperaron los valores medidos durante los muestreos en superficie (pasados 5 y 7 minutos para el buceo estacionario y la natación subsuperficial, respectivamente).

El flujo generado por el sistema fue multiplicado por las concentraciones de gases medidas para obtener la tasa instantánea de consumo de O_2 y producción de CO_2. Estas tasas instantáneas fueron integradas para obtener el volumen total de O_2 consumido y CO_2 producidos durante el periodo dentro de la cúpula. El intervalo de integración incluyó el periodo comprendido desde que la morsa se introdujo en la cúpula hasta la recuperación de los valores ambientales de O_2 y CO_2 después de que la morsa abandonase la cúpula. La $MR_{Surface}$ se calculó dividiendo el volumen total de O_2 consumido entre el tiempo que la morsa permaneció en la cúpula. Las $DMR_{Station}$ y DMR_{Swim} se calcularon dividiendo el volumen total de O_2 consumido entre el periodo del ciclo de buceo, que resulta de la suma del tiempo de inmersión y el tiempo de recuperación del buceo. Los resultados obtenidos se utilizaron para estimar cADL dividiendo la estimación del O_2 total disponible (calculado con la media de la M_b de las tres morsas) entre la media obtenida del DMR_{Swim}.

Resultados y discusión

Las tres hembras adultas de morsa del Pacífico participaron en un total de 108 sesiones de respirometría, incluyendo las sesiones de entrenamiento. Del total de muestreos, 45 fueron incluidos en el análisis (5 por animal y actividad metabólica). La $MR_{Surface}$ (4,64 ± 1,04 l O_2 min^{-1}) fue mayor que la tasa metabólica basal estimada (BMR_{est}) para un mamífero terrestre de tamaño similar, lo que concuerda con estudios previos sobre la tasa metabólica en reposo (RMR) de pinnípedos adultos. Sin embargo, aunque en el presente estudio las morsas fueron entrenadas para mantenerse en una posición relajada y tranquila durante los experimentos, los animales presentaron cierto movimiento corporal para mantener la posición requerida dentro de la cúpula. Este movimiento podría haber resultado en un valor de $MR_{Surface}$ mayor que el esperado para un animal totalmente en reposo. No obstante, el movimiento corporal mostrado por los animales podría ser similar al comportamiento habitual en su ambiente natural. Por ello, aunque los valores obtenidos de $MR_{Surface}$ podrían ser más elevados que la RMR, estos resultados podrían representar una mejor aproximación de los requerimientos energéticos de los animales en la superficie del agua en medio natural.

La $DMR_{Station}$ (3,82 ± 0,56 l O_2 min^{-1}) obtenida en el presente estudio fue menor que la $MR_{Surface}$ y la DMR_{Swim} (4,91 ± 0,77 l O_2 min^{-1}), mientras que no se encontraron diferencias significativas entre la $MR_{Surface}$ y la DMR_{Swim}. Estos resultados son similares a los obtenidos previamente en otros estudios con diferentes especies de pinnípedos, donde se mostró que la $DMR_{Station}$ es menor que la $MR_{Surface}$ o RMR. También, estudios previos en pinnípedos han mostrado que la DMR_{Swim} se encuentra por debajo o cerca de la RMR, y que se reduce conforme aumenta el tiempo de la inmersión. Este descenso en el metabolismo durante el buceo en mamíferos marinos ha sido sugerido previamente como un factor que permitiría aumentar los tiempos de buceo aeróbico en estas especies. Aunque en el presente estudio no se evaluaron los cambios en $DMR_{Station}$ y DMR_{Swim} con respecto al tiempo del buceo, la reducción del metabolismo durante el buceo explicaría los valores inferiores de $DMR_{Station}$, así como los valores similares de DMR_{Swim} con respecto a $MR_{Surface}$, a pesar del aumento del gasto energético durante la natación. Por lo tanto, los resultados obtenidos, sugieren que los buceos inactivos y la natación subsuperficial no son actividades energéticamente costosas para morsas adultas.

Por otra parte, el comportamiento (velocidad de natación y profundidad) presentado por las morsas durante los muestreos de DMR_{Swim} es similar al observado

durante comportamientos exploratorios o de tránsito en animales en el medio natural. Sin embargo, las condiciones experimentales de laboratorio no suelen ser representativas de las condiciones ambientales o de los comportamientos en el medio natural. Este hecho podría explicar que la DMR_{Swim} normalizada por la M_b en el presente estudio, fuera 2/3 inferior que la tasa metabólica medida en el medio natural en dos machos adultos de morsa del Atlántico, dado que esta aproximación habría incluido diversas actividades en circunstancias ambientales distintas. No obstante, la DMR_{Swim} obtenida podría ayudar a mejorar las estimaciones de los requerimientos energéticos durante inmersiones cortas y poco profundas en las morsas. Además, el cADL obtenido en el presente (7,7 minutos), superó el rango de las inmersiones de forrajeo más habituales en esta especie (5-6 minutos). Teniendo en cuenta los menores requerimientos energéticos resultantes de las condiciones del laboratorio, el cADL calculado en el presente estudio podría sobreestimar los valores reales en el medio natural. No obstante, la posible reducción del metabolismo con el incremento de la duración del buceo también podría conllevar un aumento del cADL. Por lo tanto, un estudio en mayor profundidad sobre los requerimientos energéticos durante buceos de diferente duración y actividad, podrá ayudar a determinar si estos animales son capaces de realizar buceos de larga duración en condiciones aeróbicas evitando la acumulación de lactato.

8.6. Conclusiones

La contribución de la presente tesis tiene un valor intrínseco dentro del campo de la fisiología de los mamíferos marinos, ya que añade información novedosa y específica sobre la fisiología respiratoria y los requerimientos energéticos de la morsa del Pacífico. Esta información básica podrá ayudar a mejorar modelos teóricos destinados a predecir las capacidades y limitaciones fisiológicas y energéticas durante el buceo en esta especie, los cuales podrán ayudar a interpretar las decisiones comportamentales de estos animales ante las condiciones cambiantes del entorno. También, la evidencia experimental obtenida en la presente tesis señala el valor potencial de la espirometría como herramienta de diagnóstico de enfermedad respiratoria en el delfín mular, proporcionando un nuevo método no invasivo para ayudar a animales bajo el cuidado humano, y en circunstancias de varamiento. Así, la contribución de la presente tesis también tendría consecuencias relevantes en la futura mejora de los esfuerzos de manejo de la fauna silvestre, y en la determinación de las prioridades de conservación. Las principales conclusiones derivadas de los Capítulos I-III de la presente tesis se destacan a continuación.

Los resultados presentados en el Capítulo I concuerdan con los observados previamente en pinnípedos y cetáceos, mostrando una capacidad de intercambio mayor (p.ej., mayor V_T y sC_L, y elevados $\dot{V}$ mantenidos durante toda la maniobra respiratoria) en la morsa del Pacífico, en comparación con los mamíferos terrestres. La evaluación de la función pulmonar en diferentes posiciones corporales en tierra y en el agua, indica una limitación al flujo en posición de decúbito ventral en tierra. Además, los resultados obtenidos sugieren que la presión hidrostática podría ayudar al retroceso elástico de los pulmones durante la maniobra espiratoria, lo que podría aumentar el flujo espiratorio en el agua, como se había sugerido previamente en leones marinos de California. Por lo tanto, los resultados presentados en este capítulo sugieren que los estudios posteriores en especies semiacuáticas deberían evaluar la función pulmonar tanto en tierra como en el agua. Además, investigaciones que evalúen la función respiratoria de individuos de diferentes edades y sexo, ayudarían a comprender el desarrollo de la fisiología y la capacidad respiratoria en esta especie.

Los resultados presentados en el Capítulo II sugieren que las PFT, o espirometrías, pueden utilizarse como una valiosa herramienta de diagnóstico para detectar enfermedades respiratorias, y para evaluar la eficacia del tratamiento en delfines entrenados para realizar espiraciones máximas mientras están en el agua. Estos resultados también indican que la espirometría podría proporcionar información adicional sobre el tipo (obstructiva o restrictiva) y la ubicación de la enfermedad respiratoria. Además, los resultados obtenidos en delfines temporalmente varados sugieren que esta herramienta no invasiva podría ayudar a definir el estado de salud respiratoria de individuos en situación de varamiento mientras respiran espontáneamente. Estudios adicionales en individuos sanos y animales con enfermedad pulmonar, podrían proporcionar valores de referencia y ayudar a relacionar los índices de función pulmonar y las curvas de flujo-volumen con diferentes tipos de enfermedades respiratorias. Estos estudios futuros podrían proporcionar un método no invasivo que ayudaría a evaluar la salud respiratoria y la eficacia del tratamiento en delfines bajo cuidado humano, así como una herramienta útil para fines de conservación in situ.

Los resultados presentados en el Capítulo III son similares a los obtenidos en trabajos previos en otras especies de mamíferos marinos, donde se observaron tasas metabólicas durante períodos de inactividad o descanso mayores que las esperadas para mamíferos terrestres de tamaño similar. Además, estos resultados coinciden con estudios

previos que mostraron una disminución de la tasa metabólica durante el buceo en comparación con períodos en la superficie. Estos resultados confirman que la morsa del Pacífico posee adaptaciones que permiten un uso eficaz del O_2 durante el buceo. Aunque esta investigación no evaluó si los requisitos energéticos disminuyen con la duración de la inmersión, como se ha observado anteriormente en otros mamíferos marinos, futuras investigaciones que estudien la inversión metabólica para inmersiones más largas ayudarían a determinar las capacidades metabólicas en esta especie. Además, los estudios que evalúen los requisitos energéticos en tierra o bajo diferentes condiciones ambientales en el agua, ayudarían a mejorar nuestra comprensión sobre el coste metabólico del comportamiento de descanso y buceo en la naturaleza. No obstante, los datos presentados en este capítulo podrán ayudar a mejorar modelos bioenergéticos destinados a cuantificar las consecuencias de los cambios ambientales en los cambios de comportamiento observados en la morsa del Pacífico. En última instancia, estos resultados junto con los obtenidos en el Capítulo I, ayudarían a comprender las limitaciones fisiológicas de esta especie, así como las limitaciones de la supervivencia de los individuos y las diferentes poblaciones de morsas.

En resumen, la presente tesis muestra el valor de estudios fisiológicos donde los individuos son entrenados para realizar procedimientos experimentales específicos mientras deciden su participación durante la recolección de datos. La desensibilización ayuda a reducir el estrés asociado a la exposición de nuevas situaciones o procedimientos. Los experimentos controlados también posibilitan la inclusión de variables que son difíciles o imposibles de controlar en la naturaleza (p. ej., edad, estado de salud, M_b, etc.). Además, la cooperación de los animales permite desarrollar nuevos equipos que pueden ser validados antes de ser utilizados con fauna silvestre. Aun así, el estudio de animales entrenados también conlleva limitaciones experimentales que deben ser consideradas (p.ej., la disponibilidad de participación puede ser variable). Las instalaciones no proporcionan condiciones representativas de los parámetros ambientales en la naturaleza (p. ej., corrientes, temperatura, profundidad, etc.), resultando en limitaciones comportamentales (p. ej., velocidad de natación, profundidad de buceo, etc.). Este hecho ha cuestionado las capacidades fisiológicas de los animales alojados bajo cuidado profesional. Sin embargo, la cooperación permite obtener datos priorizando el bienestar animal, mientras que estudios similares aplicados en el medio natural podrían conllevar un desafío logístico y ético. Por lo tanto, los estudios realizados con animales entrenados

deben diseñarse e interpretarse teniendo en cuenta sus limitaciones, donde la correcta desensibilización y la evaluación de los datos recogidos son un componente fundamental. En la presente tesis, el extenso periodo de desensibilización y entrenamiento, y la consistencia en el comportamiento observado, apoyan la publicación de los resultados obtenidos. No obstante, desentrañar la relación entre fisiología, ambiente y comportamiento en los mamíferos marinos, requiere de la combinación de información obtenida tanto en estudios de laboratorio como de campo.

8.7. Lista de símbolos y abreviaturas

- $\%VC_{PEF}$ = Porcentaje del volumen espirado durante una maniobra máxima o capacidad vital
- ADL = Límite aeróbico del buceo
- BMR_{est} = Tasa metabólica basal estimada
- cADL = Límite aeróbico del buceo estimado
- C_L = Distensibilidad pulmonar
- DMR = Tasa metabólica durante el buceo
- DMR_{Swim} = Tasa metabólica durante periodos de natación subsuperficial
- $DMR_{Station}$ = Tasa metabólica durante buceos inactivos o estacionarios
- $FEF_{25-75\%}$ = Flujo espiratorio medido entre el 25-75% de la maniobra de espiración máxima
- f_R = Frecuencia respiratoria
- M_b = Masa corporal
- $MR_{Surface}$ = Tasa metabólica en la superficie del agua
- PEF = Flujo espiratorio máximo
- PFT = Prueba de función pulmonar
- RMR = Tasa metabólica en reposo
- sC_L = Distensibilidad pulmonar específica
- s$\dot{V}$ = Flujo respiratorio normalizado por la masa corporal
- sV_T = Volumen corriente normalizado por la masa corporal
- T_{exp} = Duración de la espiración
- T_{insp} = Duración de la inspiración
- TLC = Capacidad total pulmonar
- TLC_{est} = Capacidad total pulmonar estimada
- T_{tot} = Duración total de la respiración
- $\dot{V}$ = Flujo respiratorio
- VC = Capacidad vital
- $\dot{V}_{exp}$ = Flujo espiratorio
- $\dot{V}_{insp}$ = Flujo inspiratorio
- V_T = Volumen corriente
- V_{Texp} = Volumen corriente espiratorio
- V_{Tinsp} = Volumen corriente inspiratorio

9.Appendix

Blue whale, *Balaenoptera musculus* (Linnaeus, 1758)
Australia, Indic Ocean, 2020
Photo credits: Sara Sánchez Quiñones

9. Appendix

Supplementary figures

Figure A 1. Lung function measurements in bottlenose dolphins.
Examples of lung function data collection in participating dolphins during voluntary beaching (top), and while floating at the water surface (bottom). Pictures provided by Dolphin Quest Oahu (Hawaii, USA).

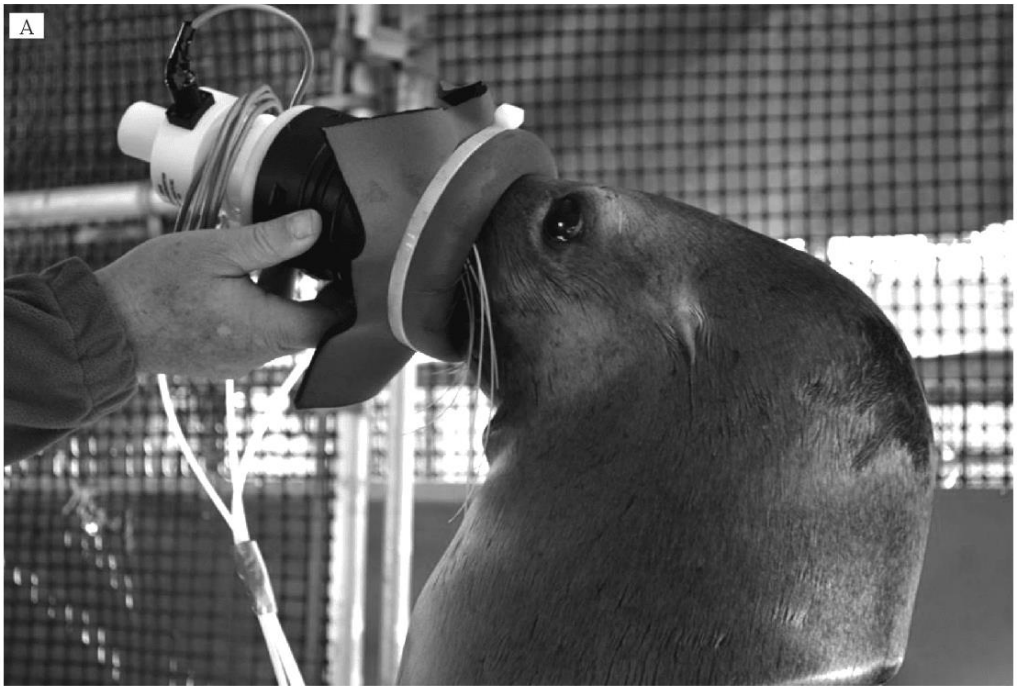
10. Annexes

Killer whale, *Orcinus orca* (Linnaeus, 1758)
Norway, Norwegian Sea, 2021
Photo credits: Joan Josep Soto Àngel

10. Annexes

Annex A: Body positions during lung function testing

Pages 70-71 from "Pulmonary function and resting metabolic rates in California sea lions (*Zalophus californianus*) on land and in water" cited as Fahlman et al., 2020b in Chapter I of the present thesis. Reproduced with permission of Aquatic Mammals Journal.



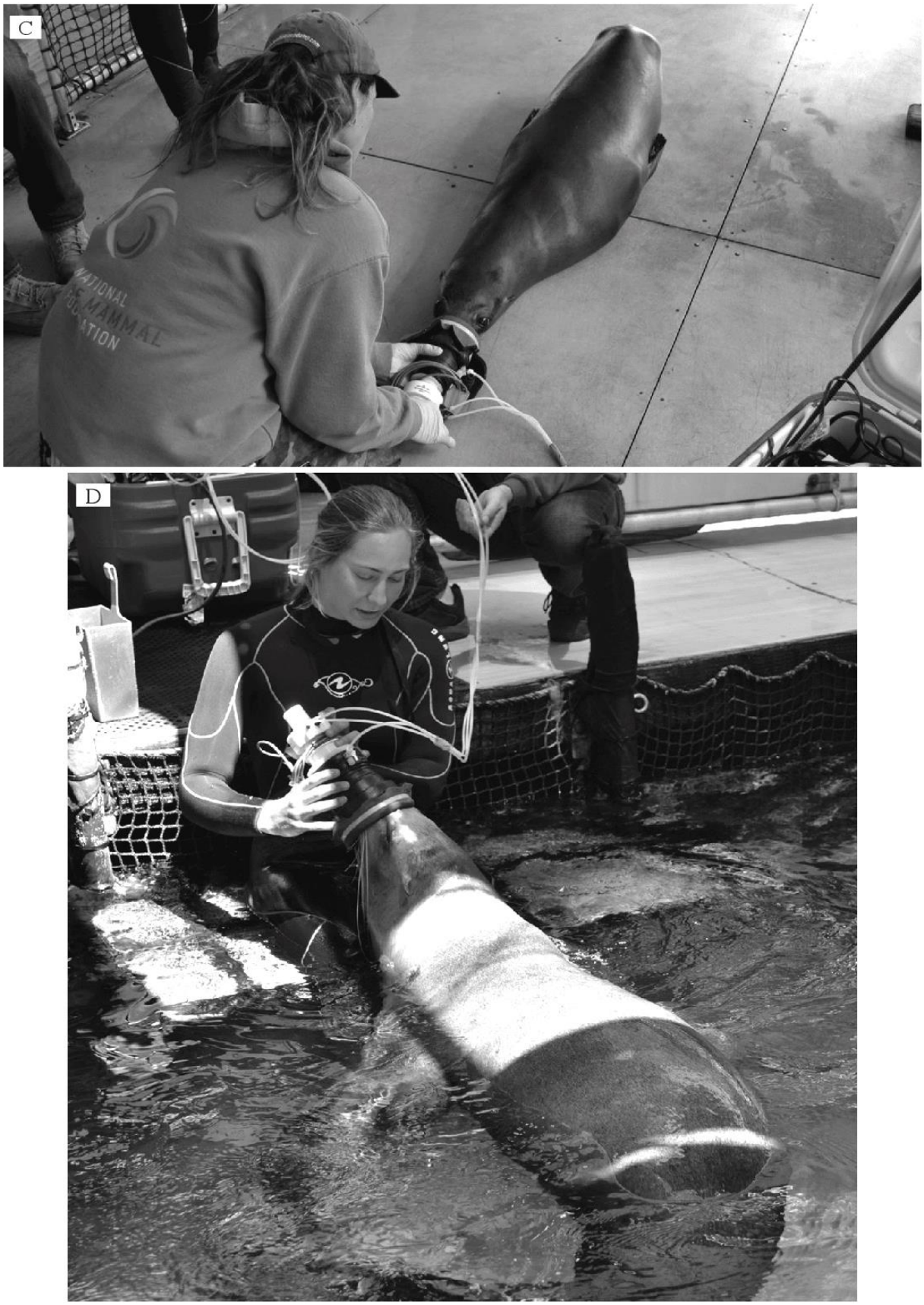


Figure 1. Images showing the (A) face mask and how breaths were measured in several positions: (B) sitting up on land, (C) laying down (sternal recumbency) on land, and (D) floating in the water.

Annex B: Lung compliance calculation

Pages 2-3 from "Respiratory function in voluntary participating Patagonia sea lions in sternal recumbency" cited as Fahlman and Madigan, 2016 in Chapter I of the present thesis.

various compartments of the respiratory system to the limit of collapse. The results from the model suggested that the diving lung volume, the relative size of the upper (conducting airways) and lower (alveoli) airways, and the structural properties (compliance) of the upper and lower airways were important in determining the depth at which the alveoli collapse and gas exchange ceases (Bostrom et al., 2008). The model output was compared to available data for collapse depth in different species (Kooyman et al., 1970, 1972; Ridgway and Howard, 1979; Kooyman and Sinnett, 1982; Falke et al., 1985), and it was concluded that behavioral (diving lung volume) and structural (lung and dead space compliance) variations between species could account for the observed species differences (Bostrom et al., 2008; Fahlman et al., 2009).

Static pressure-volume loops are commonly used to measure the physical properties (compliance) of the respiratory system. Published data exist on excised lungs for several terrestrial species, but only a few measurements have been made for marine mammals (Denison et al., 1971; Piscitelli et al., 2010; Fahlman et al., 2011; Moore et al., 2014). A recently published theoretical study compared estimated blood and tissue gas tensions (O_2, CO_2, and N_2) using species-specific respiratory compliance estimates or those previously published from terrestrial mammals (Hodanbosi et al., 2016). The results showed that the blood and tissue PN_2 levels were significantly lower using the species-specific compliance estimates, while there were little or no differences for blood and tissue PCO_2 or PO_2 levels. It is therefore difficult to assess the accuracy of the model output without species-specific respiratory compliance estimates. In addition, common indices of respiratory capacity and mechanics, e.g., lung compliance, inspiratory and expiratory tidal volume, flow-rate, and duration, exist for a limited number of marine mammals of different age classes (Parker, 1922, 1932; Scholander and Irving, 1941; Robin et al., 1963; Olsen et al., 1969; Kooyman et al., 1971; Leith, 1976; Gallivan, 1981; Kooyman and Cornell, 1981; Gallivan et al., 1986; Bergey and Baier, 1987; Reed et al., 1994, 2000; Fahlman et al., 2015). Only by acquiring comparable data from multiple species, can one begin to understand the level of confidence with which one can predict such parameters for species where we have little or no data. The present study was undertaken to extend the current knowledge of the respiratory physiology and mechanical properties of Patagonia sea lions (*Otaria flavescens*) participating voluntarily. In addition, we also collected end-expiratory gases and estimated breath-by-breath gas exchange. Our results indicate that the metabolic demand and respiratory physiology of Patagonia sea lions are similar to other pinniped species.

MATERIALS AND METHODS

Animals and Morphometrics

Five Patagonia sea lions (*O. flavescens*), 3 females and 2 males, managed under human care participated under voluntary control (**Table 1**). All work was approved by the IACUC at Texas A&M University—Corpus Christi (TAMUCC-IACUC AUP # 04-11).

Each animal was weighed (±0.5 kg) before the start of each procedure (**Table 1**).

Flow Measurements

Ventilatory flow-rates were measured using a pneumotachometer (3813 series, 0–800 l min^{-1}, Hans-Rudolph Inc., Shawnee, KS) placed inside a modified anesthesia face-mask. The maximum dead-space of the mask was 200 mL, and varied slightly depending on how much of the snout was placed inside the mask. The pneumotachometer was connected to a differential pressure transducer (MPX-2.5 mbar type 339/2, Harvard apparatus, Holliston, MA) via a 310 cm length of 2 mm I.D, firm walled, flexible tubing. The pneumotachometer was calibrated for linearity and flow using a 7 L calibration syringe (Series 4900, Hans-Rudolph Inc., Shawnee, KS) immediately before and after each trial, through a series of pump cycles at various flow rates. The pump cycles allowed the relationship between differential pressure and flows for the expiratory and inspiratory phases to be determined. To avoid spurious peaks, the reported maximal inspiratory and expiratory flows are the average flows over 20 ms; 10 ms on either side of the maximal recorded inspiratory or expiratory flow.

Airway and Esophageal Pressures

Airway pressure (P_{aw}) was measured via a sample port immediately above the nostrils connected to a differential pressure transducer (MPX-100 mbar type 339/2, Harvard apparatus, Harvard Apparatus, Holliston, MA). Esophageal pressure (P_{eso}) was measured using an esophageal balloon catheter (47–9005, Cooper Surgical, Trumbull, CT) connected to a differential pressure transducer (MPX-100 mbar type 339/2, Harvard apparatus, Holliston, MA) by a 288 cm length of 2 mm I.D., firm walled, flexible tubing, through a 3 way stopcock. The balloon catheter was manually inserted into the esophagus to the approximate level of the heart, and inflated with 1.0 ml of air (**Figure 1**). Reference pressure for both P_{ao} and P_{eso} was ambient atmospheric pressure (P_{amb}).

Data Acquisition of Differential Pressures

Differential pressure transducers were connected to an amplifier (Tam-A, Harvard apparatus, Holliston, MA). The data from the transducers were captured at 400 Hz using a data acquisition system (Powerlab 8/35, ADInstruments, Colorado Springs, CO), and displayed on a laptop computer running LabChart (v. 8.1, ADInstruments, Colorado Springs, CO). All differential pressure transducers were zeroed immediately before each trial.

Dynamic Responses

The dynamic response of the system configured as described above was evaluated for a step response by a balloon deflation test. Popping an inflated balloon with a needle provided an immediate step change in pressure. The time constant (τ) was estimated as the time to 50% pressure reduction, or $\tau_{1/2}$. The dynamic constant was 7 ms for the pneumotachometer pressure line and 40 ms for the esophageal catheter.

Respiratory Gas Composition

Respiratory gasses were subsampled via a port in the pneumotachometer and passed through a 310 cm length of 2 mm I.D., firm walled, flexible tubing and a 30 cm length

TABLE 1 | Animal ID, sex [female (F)/male(M)] age (years), body mass (M_b, kg), rate of O_2 consumption ($\dot{V}O_2$, superscript number is the number of repeated measurements of at least 3 min), and CO_2 production ($\dot{V}CO_2$, number of measurements are the same as for $\dot{V}O_2$), breathing frequency with mask on (f_{Rm}, breaths min^{-1}) or during a 5 min focal observation (f_{Rf}), and lung compliance (C_L) of 5 Patagonia sea lions (*Otaria flavescens*) participating in the study.

Animal ID	Sex	Age	M_b	$\dot{V}O_2$	$\dot{V}CO_2$	f_{Rm}	f_{Rf}	C_L
		Yr	(kg)	(L min^{-1})	(L min^{-1})	bpm		L cmH_2O^{-1}
Sayula	F	14	113.0 ± 4.2	0.695 ± 0.162^{11}	0.510 ± 0.145	4.6 ± 1.8^{9}	4.1 ± 0.3^{3}	0.22 ± 0.08^{50}
Jenny	F	14	100.5 ± 2.1	0.840 ± 0.234^{10}	0.637 ± 0.194	5.0 ± 2.1^{10}	8.3 ± 0.2^{3}	0.23 ± 0.06^{80}
Rex	M	5	143.5 ± 4.9	1.514 ± 0.361^{14}	1.235 ± 0.169	3.8 ± 0.9^{8}	4.1 ± 0.2^{3}	0.49 ± 0.16^{82}
Mara	F	14	94.3 ± 4.6	0.881 ± 0.123^{7}	0.729 ± 0.209	5.1 ± 1.2^{9}	4.6 ± 1.3^{3}	0.31 ± 0.12^{180}
Nano	M	15	286	N/A	N/A	3.3 ± 0.9^{3}	3.6 ± 0.7^{3}	N/A

Superscript numbers is the number of repeated measurements for that variable.

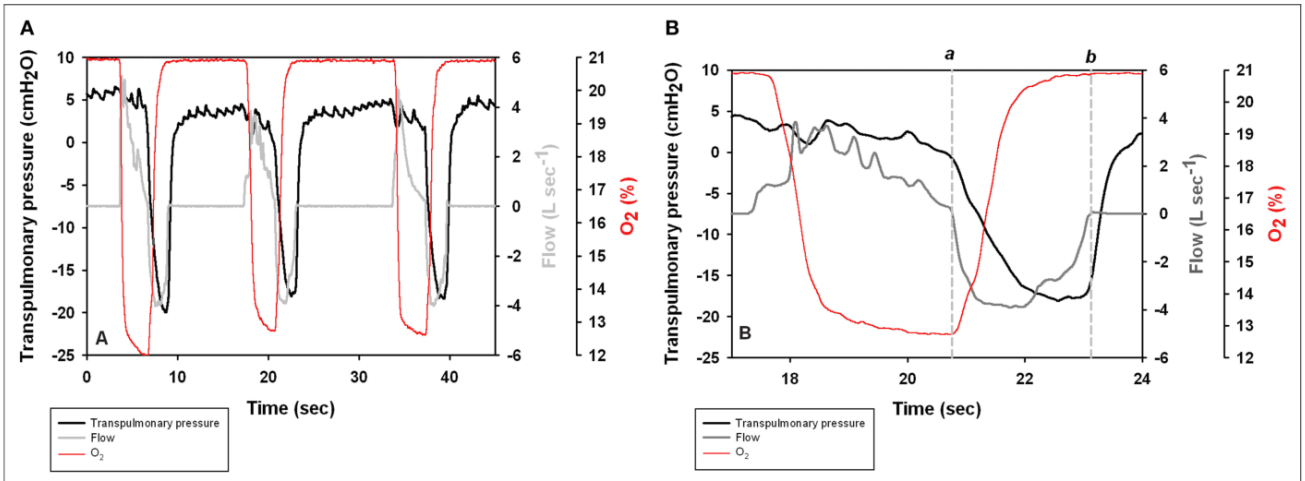


FIGURE 1 | (A) Transpulmonary pressure ($P_{tp} = P_{ao}–P_{eso}$) pressure, respiratory flow, and O_2 content during three voluntary breaths in a Patagonia sea lion. Positive values represent exhalation. **(B)** Expanded scale shows the spontaneous breath with times of 0 flow (vertical lines a and b) used to estimate lung compliance.

of 1.5 mm i.d. Nafion tubing, to fast-response O_2 and CO_2 analyzers (ML206, Harvard Apparatus, Holliston, MA, USA) at a flow rate of 200 ml min^{-1}. The gas analyzers were connected to the data acquisition system and sampled at 400 Hz. The gas analyzers were calibrated before and after the experiment using a commercial mixture of 5% O_2, 5% CO_2, and 90% N_2, certified accurate to at least 0.01%. (Gasco, Oldsmar, FL. Prod#17L-340). Mean daily air temperature, humidity, and ambient pressure were 29.7 ± 2.9°C (range 20.1–33.6°C), 89.9 ± 13.8% (range 57–100%), and 101.0 ± 0.3 kPa (range 100.7–101.4 kPa, $n = 32$ trials).

Lung Compliance

Lung compliance ($C_L = VT \bullet \Delta P_{tp}^{-1}$, L $\bullet cmH_2O^{-1}$) was estimated as the inspiratory tidal volume divided by the change in transpulmonary pressure ($\Delta P_{tp} = \Delta P_{aw} - \Delta P_{eso}$), measured at zero flow (Olsen et al., 1969; Fahlman et al., 2015). The esophageal pressure trace was phase corrected by 40 ms (the dynamic response time of the esophageal catheter system), and P_{eso} was determined at the points of zero flow of the expiratory phase and inspiratory phases (a and b, respectively, in Figure 2C in Fahlman et al., 2015).

Metabolic Rates

The respiratory gas data were phase corrected to account for the delay caused by the flow in the sample line. The expiratory flow-rate and expired O_2 content was multiplied to calculate the instantaneous $\dot{V}O_2$. The instantaneous $\dot{V}O_2$ was integrated over each breath to yield the total volume of O_2 exchanged during each breath. The volume was summed for each trial period and divided by the duration of the trial to provide an estimate of the oxygen consumption rate for that time period.

To account for differences in body mass, $\dot{V}O_2$ was converted to mass-corrected metabolic rate ($s\dot{V}O_2$) using the previously predicted mass exponent for Steller sea lions (body $mass^{0.6}$; McPhee et al., 2003).

Data Processing and Statistical Analysis

All gas volumes were converted to standard temperature pressure dry (STPD, Quanjer et al., 1993). Exhaled air was assumed

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