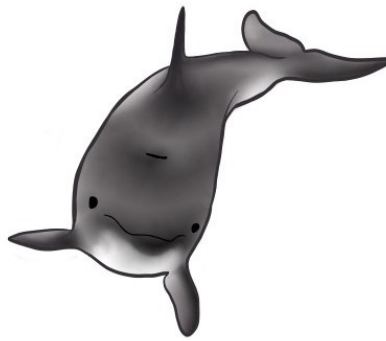
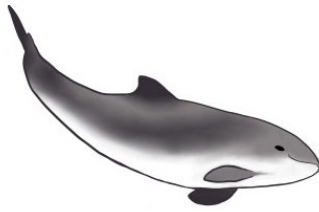
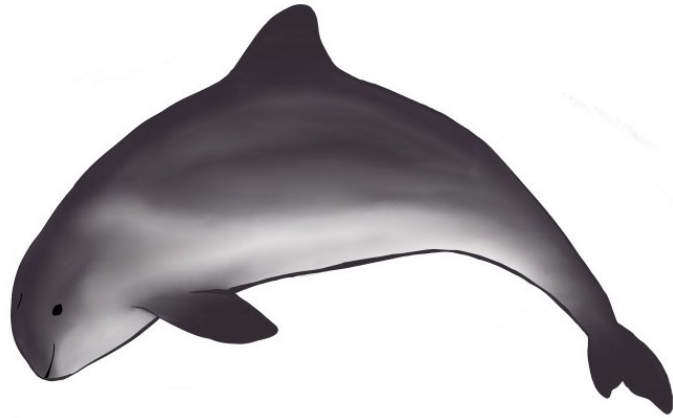


**Molecular and pathophysiological responses
in non-healthy and healthy
harbour porpoises (*Phocoena phocoena*)**



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

Jacques Cousteau

**Molecular and pathophysiological responses
in non-healthy and healthy
harbour porpoises (*Phocoena phocoena*)**

Dissertation

in fulfilment of the requirements for the degree of Doctor of Natural Science

(Doctor rerum naturalium, Dr. rer. nat.)

submitted by

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Manuscript (including all authors, title, and if applicable journal, year; mark all first authors with asterisk):

Dönmez EM*, Siebert U, Fabrizio A. 2023. Diving on Damage – the muscle transcriptome of parasitic infested harbor porpoises (*Phocoena phocoena*) hints at oxidative stress but not hypoxia. *Frontiers in Marine Science* 10, 1232305.

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Preparation of a sample protocol for tissue collection
Assistance in subsampling

Method development:

Data generation and analyses (including contribution to specific figures):

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Conduction of the quantitative real-time PCR (qRT-PCR), including primer design and cDNA synthesis
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Data interpretation and data visualisation

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Dönmez EM, Drotleff B, Siebert U, Fabrizius A. Insights from untargeted metabolomics of wild lung-diseased harbour porpoises. **Poster** at the 36th European Cetacean Society Conference – Ponta Delgada, Portugal, 12-1609.11.2024 May 2025

Dönmez EM, Siebels B, Nissen P, Siebert U, Fabrizius A. From the lung to the muscle how integrative MultiOmics analyses shed light on new possible molecular adaptations of harbour porpoises. **Oral talk** at the 25th Biennial Conference of the Biology of Marine Mammals - Perth, Australia, 09-15 November 2024

Dönmez EM, Siebels B, Nissen P, Siebert U, Fabrizius A. Integrative MultiOmics approach reveals a possible adaptation of harbour porpoise lung tissue as response to damage. **Oral talk** at the Annual Conference of the Society of Experimental Biology - Prague, Czech Republic, 2-5 July 2024

Dönmez EM, Siebert U, Fabrizius A. Extensive immune reaction and highly expressed novel genes hint at a potentially molecular adaptation in the lung of non-healthy harbour porpoises. **Poster** at the 35th European Cetacean Society Conference - Catania, Italy, 08-12 April 2024

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Dönmez EM, Siebert U, Fabrizius A. Diving on Damage – Molecular adaptations in the lung and muscle of parasitic infested harbour porpoises (*Phocoena phocoena*). **Poster** at the 114th Annual Meeting of the German Zoological Society 2022 - Bonn, Germany, 12-16 September 2022

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2 Summary

Harbour porpoises (*Phocoena phocoena*) in the North and Baltic Seas are constantly exposed to cumulative environmental and anthropogenic stressors that compromise their health. In particular, pathological lesions in the lungs that are caused or exacerbated by accumulating nematode infestations are a serious health issue. Such damages may not only impair respiratory function, but may also reduce oxygen delivery to end-organs, such as the skeletal muscles. Ultimately, this may negatively affect diving ability, resilience and overall fitness with potential population-level consequences.

The aim of this dissertation was to investigate whether harbour porpoises with severe lung nematode infestations and associated secondary diseases possess molecular mechanisms to tolerate these pathological and parasitic insults to the respiratory system and whether the pathophysiological effects extend systemically to the skeletal muscles. To achieve this, lung and muscle tissue samples of harbour porpoises that died from stranding or as by-catch were used, and analysed by a combined omics approach, including transcriptomics, proteomics, metabolomics and lipidomics.

Transcriptomic profiling and quantitative real time-PCR (qRT-PCR) were used to assess potential molecular changes in the muscles that may be indicative of reduced oxygen supply as a consequence of the pathological lung (**Chapter I**). The muscles showed signs of increased antioxidant activity, indicating elevated oxidative stress, but not a typical hypoxia response. Elevated transcripts involved in lipid metabolism and glycolysis pointed to energetic imbalance. A tightly regulated dynamic between tissue repair and turnover may be indicated by enhanced expression of transcripts related to regeneration and selective protein degradation, thus may support muscle maintenance despite ongoing stress exposure. These findings provided initial molecular insights that declining lung health may result in downstream effects in harbour porpoises.

In **Chapter II**, the pathophysiological response in the lungs of non-healthy harbour porpoises was examined by using comparative transcriptomics, qRT-PCR, and *de novo* transcriptome assembly. The gene expression in the lungs of non-healthy individuals indicated a proinflammatory immune response, pointing to a persistent or exacerbating, and potentially compromising immune activation, underscoring findings of published studies. The similar regulation compared to humans suffering from lung diseases suggested that pulmonary immune responses may be conserved among mammals despite different life histories and habitats.

Shared dysregulated expression patterns between the lungs and muscles indicated potential whole-organism consequences. The identified, significantly dysregulated non-coding RNAs via the *de novo* assembly may further regulate adaptive immunity, warranting future investigation to determine their function.

Chapter III presents the integration of multiple omics datasets to obtain comprehensive insights into molecular processes across lungs and muscles in non-healthy harbour porpoises. Lungs of non-healthy porpoises displayed elevated antioxidant and anti-inflammatory features, including plasmalogen lipids, pointing to increased oxidative damage. Furthermore, dysregulation of immune responses underscored the results in Chapter II and indicated a chronic, systemic inflammation. Reduced collagen and increased cardiolipin levels in the lungs of non-healthy porpoises hinted at progressive tissue damage and impaired tissue function. In the skeletal muscles, reduced lipid metabolism and disrupted tissue maintenance suggested exceeding energy demand that may accelerate muscle catabolism and atrophy. Molecular alterations shared by both tissues supported a potential systemic impairment originating from the respiratory system. The integration of the omics data enabled the identification of potential biomarker panels, including proteins, metabolites, and lipids, which may support health monitoring of free-ranging harbour porpoises after validation in functional studies with larger sample sizes.

Collectively, this dissertation reveals that respiratory disease and accumulating parasite infestations in free-ranging harbour porpoises extend beyond localised pulmonary damage to a systemic condition that affects immunity, energetic balance, and tissue maintenance. By linking declining pulmonary health with systemic stress, it highlights functional consequences and energetic trade-offs that may become critical for harbour porpoises under intensifying anthropogenic pressures and shifting ocean conditions. The identified molecular biomarker panels may support future non-invasive health monitoring and emphasise the growing potential of emerging omics technologies for cetacean health research. Together, these findings advance the understanding of how disease and anthropogenic pressures may shape the health, resilience and survival of harbour porpoise populations in the North and Baltic Seas.

3 Zusammenfassung

Schweinswale (*Phocoena phocoena*) in der Nord- und Ostsee sind kontinuierlich kumulativen umweltbedingten und anthropogenen Stressfaktoren ausgesetzt, die ihre Gesundheit beeinträchtigen können. Insbesondere pathologische Läsionen in der Lunge, die durch den verstärkten Befall mit parasitären Nematoden verursacht oder verschlimmert werden, sind ein ernstzunehmendes Gesundheitsproblem. Solche Schädigungen können nicht nur die Lungenfunktion beeinträchtigen, sondern auch die Sauerstoffversorgung von Endorganen, wie den Skelettmuskeln, verringern. Dies kann sich letztendlich negativ auf die Tauchfähigkeit, die Widerstandsfähigkeit und die allgemeine Fitness auswirken und in potenziellen Konsequenzen auf Populationsebene resultieren.

Ziel dieser Dissertation war es zu untersuchen, ob Schweinswale mit schwerwiegendem Lungenwurmbefall und damit assoziierten Folgeerkrankungen über molekulare Mechanismen verfügen, um diese pathologischen und parasitären Beeinträchtigungen des Respirationssystems zu tolerieren, und ob sich die pathophysiologischen Auswirkungen systemisch auf die Skelettmuskulatur ausweiten. Hierfür wurden Lungen- und Muskelgewebeproben von gestrandeten oder als Beifang verendeten Schweinswalen verwendet, und durch einen kombinierten Omics-Ansatz mittels Transkriptomik, Proteomik, Metabolomik und Lipidomik analysiert.

Mithilfe von Transkriptom-Analysen und quantitativer real time-PCR wurde untersucht, ob erkrankte Schweinswale molekulare Veränderungen aufzeigen, die auf eine reduzierte Sauerstoffversorgung infolge der Lungenschädigungen hindeuten könnten (**Kapitel I**). Die Muskeln zeigten Anzeichen einer erhöhten antioxidativen Aktivität, die auf einen erhöhten oxidativen Stress hindeutete, jedoch nicht auf eine typische Hypoxie-Antwort. Vermehrt vorhandene Transkripte, die am Fettstoffwechsel und an der Glykolyse beteiligt waren, deuteten auf ein Ungleichgewicht des Energiehaushalts hin. Ein streng reguliertes Verhältnis zwischen Gewebereparatur und -umsatz könnte durch eine verstärkte Expression von Transkripten im Zusammenhang mit Regeneration und selektivem Proteinabbau angedeutet sein, und den Muskelerhalt trotz anhaltender Belastung unterstützen. Diese Ergebnisse lieferten erste molekulare Erkenntnisse darüber, dass eine verminderte Lungengesundheit bei Schweinswalen weitreichende Auswirkungen haben könnte.

In **Kapitel II** wurde die pathophysiologische Antwort in den Lungen erkrankter Schweinswale mittels vergleichender Transkriptomik, qRT-PCR und einer *De novo*-Transkriptom-

Assemblierung untersucht. Die Genexpressionsmuster in erkrankten Individuen deuteten auf eine proinflammatorische Immunantwort hin, welche auf eine anhaltende oder sich verschlimmernde, sowie potenziell kompromittierte Immunaktivierung hinweisen könnte und die Ergebnisse veröffentlichter Studien bekräftigt. Die Ähnlichkeit der Regulation zu der in Menschen mit Lungenkrankheiten lässt vermuten, dass pulmonale Immunantworten in Säugetieren trotz unterschiedlicher Lebensumstände und -räume konserviert sind. Gleichartig dysregulierte Expressionsmuster zwischen Lunge und Muskeln deuteten auf potenzielle Konsequenzen für den gesamten Organismus hin. Die signifikant dysregulierten nicht-kodierenden RNAs, die durch die *De novo*-Assemblierung identifiziert wurden, könnten ebenfalls die adaptive Immunabwehr regulieren und erfordern weitere Untersuchungen zur Bestimmung ihrer Funktion.

Kapitel III beschreibt die Integration mehrerer der Omics-Datensätze, um einen umfassenden Einblick in die molekularen Prozesse in Lungen und Muskeln von erkrankten Schweinswalen zu erhalten. Lungen von befallenen Schweinswalen wiesen erhöhte antioxidative und entzündungshemmende Eigenschaften auf, unter anderem Plasmalogen-Lipide, was auf eine zunehmende oxidative Schädigung hindeuten könnte. Zudem unterstützte die Dysregulation der Immunantworten die Ergebnisse aus Kapitel II und wies auf eine chronisch-systemische Entzündungsreaktion hin. Reduziertes Kollagen und erhöhtes Kardiolipin in den Lungen von erkrankten Schweinswalen ließen auf progressive Gewebeschäden und eine eingeschränkte Gewebefunktion schließen. In den Skelettmuskeln deuteten ein verminderter Fettstoffwechsel und eine beeinträchtigte Gewebeerhaltung auf einen überhöhten Energiebedarf hin, der den Katabolismus und die Atrophie vom Muskel beschleunigen könnte. Molekulare Veränderungen, die in beiden Geweben ähnlich waren, stützten die Annahme, dass eine systemische Beeinträchtigung sowie Auswirkungen auf den gesamten Organismus vorliegen, die vom Atmungssystem ausgehen. Die Integration der Omics-Daten ermöglichte die Identifizierung potenzieller Biomarker-Panels, die Proteine, Metaboliten und Lipide umfassen. Diese könnten nach der funktionellen Validierung in einer größeren Stichprobe das Gesundheitsmonitoring von frei-lebenden Schweinswalen unterstützen.

Diese Dissertation verdeutlicht, dass Lungenkrankheiten und zunehmender parasitärer Befall bei frei-lebenden Schweinswalen über lokalisierte Lungenschädigungen hinausgehen und zu einem systemischen Problem führen, der das Immunsystem, den Energiehaushalt und den Gewebeerhalt beeinträchtigt. Der Zusammenhang zwischen der Verschlechterung der Lungengesundheit und dem systemischen Stress zeigt die funktionellen Folgen und

energetischen Kompromisse auf, die für Schweinswale unter wachsendem, anthropogenem Druck und sich verändernden Meeresbedingungen kritisch werden könnten. Die identifizierten, molekularen Biomarker-Panels könnten künftig nicht-invasives Gesundheitsmonitoring unterstützen und unterstreichen das Potenzial moderner Omics-Technologien für die Erforschung der Gesundheit von Walen. Diese Erkenntnisse tragen zu einem verbesserten Verständnis bei, wie Krankheiten und anthropogene Stressfaktoren die Gesundheit, Widerstandsfähigkeit und das Überleben von Schweinswalpopulationen in der Nord- und Ostsee beeinflussen können.

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4 General Introduction

4.1 A small cetacean in the North and Baltic Seas

Few marine mammal species are endemic to the German North and Baltic Seas. Among them are the harbour, ringed and grey seals from the pinniped family, as well as various cetacean species (ASCOBANS, 2002; Hammond et al., 2017; Kauhala et al., 2019; Kinze et al., 2021; Lakemeyer et al., 2020). While infrequent sightings and strandings of several other marine mammals happen, the harbour porpoise is the only cetacean recognised as resident, and is widely abundant in the North and Baltic Seas (Fig. 1A; Gilles et al., 2023; Hammond et al., 2017; Siebert et al., 2006b; Wenger & Koschinski, 2012).

Harbour porpoises belong to the smallest cetaceans and have a rather short lifespan of up to 12-20 years (Kesselring et al., 2017; Lockyer, 2003; Read & Hohn, 1995). Mostly coast-dwelling, the harbour porpoise opportunistically hunts benthic and pelagic prey sources (Heße et al., 2025; Schaffeld et al., 2016). However, according to their localisation and seasonal availability, they tend to show preference for some species, such as the Atlantic herring (Andreasen et al., 2017; Blanchet et al., 2019; Hammond et al., 2017). Due to a large surface-to-body ratio and habitat in temperate waters, harbour porpoises are prone to high energy losses through heat radiation and conduction (Kleiber, 1947). Together with their high metabolic field rate, this results in an increased energetic demand which requires harbour porpoises to constantly locate prey and feed to uphold insulation and basic functions (Rojano-Donãte et al., 2018; Wisniewska et al., 2016). Their generally shy nature, in addition to their hardly visible blow and small stature, makes them challenging to observe at the surface. They are known to spent little time at the sea surface and largely remain at depths around 30 m (Westgate et al., 1995), though telemetry studies have recorded dives up to 400 m deep (Nielsen et al., 2018; Westgate et al., 1995). As shallow divers, they dive repeatedly for short durations and tend to only prolong their dives when encountering a promising prey patch (up to 179 dives/hour, 6-321 s long; Linnenschmidt et al., 2013; Otani et al., 2001; Westgate et al., 1995). Harbour porpoises depend on sound perception and echolocation for foraging, orientation and communication (Sørensen et al., 2018; Verfuß et al., 2005, 2009; Wisniewska et al., 2018). Their clicks are pulsed and high frequency, thus do not travel far (Carlström, 2005; Cucknell et al., 2017). Due to their elusive lifestyle, monitoring, observation and management of these animals is challenging and not easy to facilitate. Three distinct populations are recognised: the North Sea, the Belt and the Baltic Proper population (Fig. 1B; Galatius et al., 2012; Lah et al., 2016; Tiedemann et al.,

1996; Wiemann et al., 2010). While they are of least concern on a global scale, their abundance in the North Sea shows a declining trend ($\sim -1.80\%$ per year, estimated 339000 individuals; Gilles et al., 2023; Nachtsheim et al., 2021) and both Baltic Sea subpopulations are listed as critically endangered or endangered (Belt: est. 14400 individuals; Baltic Proper: est. < 500 individuals; Benke et al., 2014; Carlström et al., 2023; Gilles et al., 2023; HELCOM, 2013). Consequently, harbour porpoises are strictly protected under various laws, and national and international conventions in Europe (EU Habitats Directive, 1992; HELCOM, 2013; ASCOBANS¹), and EU states are obligated to report on their population status.

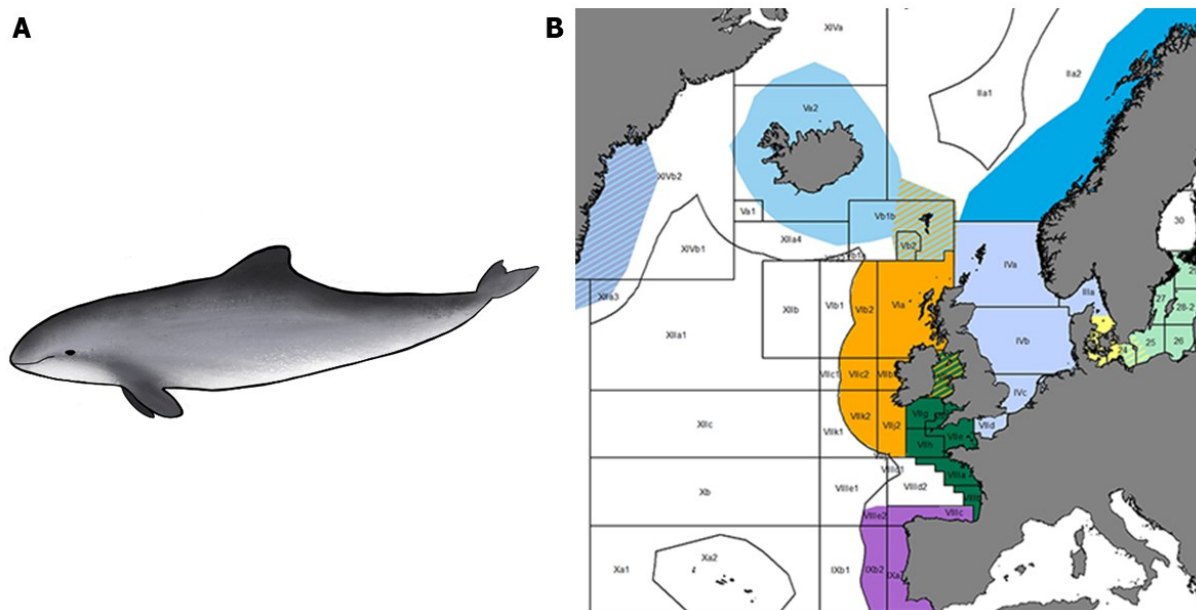


Figure 1 Harbour porpoise and its distribution and habitat of the harbour porpoise in the European part of the North Atlantic. **A** Illustration of a harbour porpoise (illustrated by Eda Dönmez). **B** The habitat of the harbour porpoise populations in the seas adjacent to Germany are highlighted in light blue-grey (Greater North Sea), yellow (Belt Sea) and light green (Baltic Sea). Sections in the map represent the transects of the NAMMCO and IMR 2019 survey. Modified from NAMMCO (2019)

4.2 Harbour porpoises as indicator species of ecosystem health

Long-term monitoring and health assessments are a key tool to gain essential insights into the structure, vulnerability and resilience of wildlife populations. Harbour porpoises belong to the long-lived sentinel species, thus are exposed to a complex web of stressors over their lives, and sit on top of the trophic level as apex predators. As such, changes in their abundance or population health can reflect the impact of critical environmental changes and can be indicative of ecosystem health, such as prey species fluctuations, bioaccumulation of contaminants and habitat degradation (Bossart, 2011; Williams et al., 2020). Wildlife monitoring therefore is not

¹ www.ascobans.org

only integral to assess the resilience and vulnerability of populations, but is also essential to observe changes in the environment and to evaluate overall ecosystem stability. As harbour porpoises are cryptic, mobile and fully aquatic marine mammals, monitoring and health assessment efforts are associated with profound logistical and conservational challenges. Within the German jurisdiction, handling, disturbing or sampling free-ranging individuals is prohibited, thus monitoring schemes rely on passive acoustic monitoring, ship-based and aerial surveys, and the summarised findings of routinely performed post-mortem examinations (necropsies; Gilles et al., 2025; Nachtsheim et al., 2021; Scheidat et al., 2024; Siebert et al., 2020). While visual and acoustic monitoring can help to estimate abundance, distribution and movement patterns, the findings of post-mortem examinations allow the collection of a plethora of information, such as age, nutrition and reproductive status, sex, and the impact of environmental or anthropogenic pressures. In addition to enabling comprehensive long-term trends in population structure and health, necropsy data is a key source to detect the emergence of infectious diseases or parasitic species (IJsseldijk et al., 2022, 2024; Lennon et al., 2025). Infectious diseases and parasitic infestations can considerably affect mortality, impact population structure and recovery, and are among the leading causes of death in cetaceans (Siebert et al., 2001; Van Bressem et al., 2009; Vigil et al., 2024). Therefore, a comprehensive understanding of cetacean health is crucial to develop effective methods and tools for the early detection of potential impacts, particularly within endangered and vulnerable populations.

4.3 Prevalence, pathophysiology, and host-pathogen dynamics of lung nematodes

4.3.1 Lung nematode infestations in harbour porpoises

Harbour porpoises of the North and Baltic Seas suffer from progressively worsening health and often host a plethora of parasitic communities (Schick et al., 2020; Siebert et al., 2020; Sonne et al., 2020b). Endoparasites, such as nematodes, are a frequent pathological finding of necropsies (Siebert et al., 2001, 2006a, 2020). Common nematode species include *Pseudalius sp.*, *Halocercus sp.* and *Torynurus sp.*, which primarily colonise the respiratory tract and often co-occur within different compartments of the pulmonary system (Jepson et al., 2000; Lehnert et al., 2014; Measures, 2001). Previous studies on harbour porpoises have consistently highlighted the respiratory tract as a key organ for assessing health status (Lennon et al., 2025;

Siebert et al., 2020), reflecting both its essential function in cetaceans and its susceptibility to pathogens and parasites.

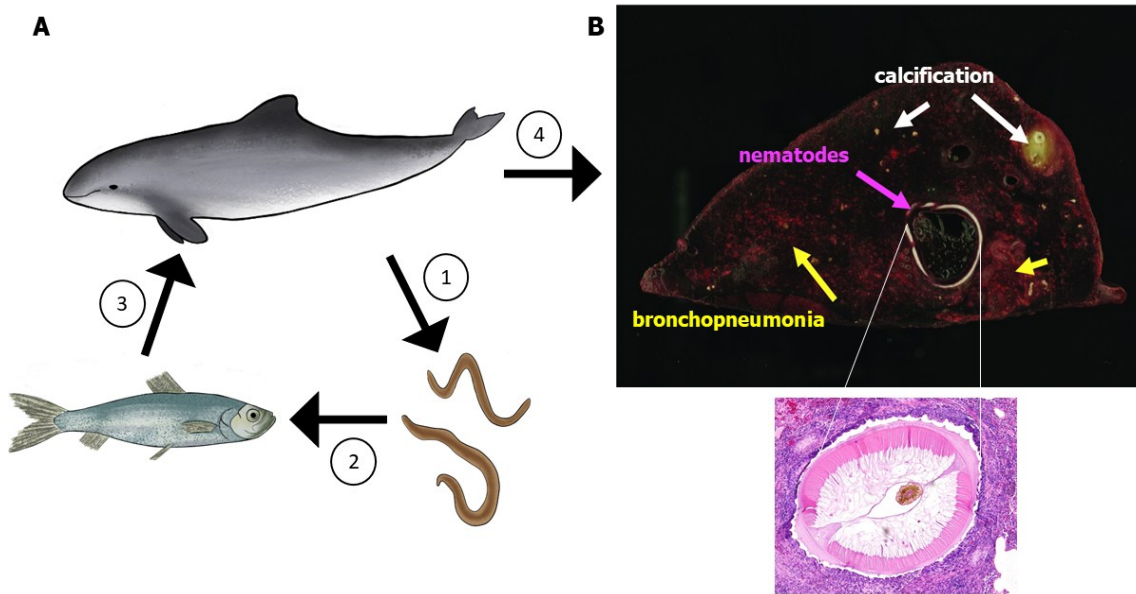


Figure 2 Life cycle of lung nematodes and pulmonary impacts. **A** Lung nematodes are regularly found in the respiratory tract of harbour porpoises and are distributed as larvae through faeces (1). L1 larvae are then ingested by intermediate hosts, such as fish (e.g., Atlantic herring), in which they grow to the L3 stage (2). Via feeding on infected intermediate hosts, L3 larvae get transmitted to harbour porpoises, their final host, and develop into adults (3). Nematode infestations and pulmonary nematodiasis result in pathological lung lesions. **B** Within the respiratory tract, lung nematodes feed on surrounding tissue and can block blood circulation by moving through blood vessels (pink arrows). Nematodes and parasitic molecules and debris can cause tissue damage, calcifications (white arrow) or fibrosis. High parasitic burden also affects immune competence and can promote the susceptibility or exacerbate contracted secondary bacterial or viral diseases, such as bronchopneumonia (yellow arrows). The small histological staining shows a lung nematode and surrounding chronic bronchitis (40x magnification, both images of B are modified from Siebert et al., 2020, used under CC-BY-NC-ND). Illustrations by Eda Dönmez

Initial transmission of lung nematodes occurs by ingestion of infected prey, such as fish (Fig. 2A), which serve as intermediate hosts (Dailey, 1970). As a result, accumulation of parasites is thought to worsen with age and increased food intake, as more larval stages are ingested and can develop to adults within the harbour porpoise. Vertical transmission from the mother to the calf, either through the placenta or mammary, has also been considered, as lung nematode presence has been observed in neonates (Balbuena et al., 1994). Although the significance of lungworm burden for the health of harbour porpoises is still debated (Kirkwood et al., 1997; van Elk et al., 2019), the prevalence and intensity of parasite load have increased over the past decades (Lehnert et al., 2014; Siebert et al., 2020; Ten Doeschate et al., 2017). While the prevalence of lungworms is higher in Scandinavian populations (< 80%), individuals often suffer from mild infestations (Lehnert et al., 2014; Siebert et al., 2006a). In the North and Baltic Seas, lungworms are present in around half of the examined individuals, with infestations often being more severe and associated with increased pathological lesions, disease susceptibility, and mortality (Baker & Martin, 1992; Jepson et al., 2000; Siebert et al., 2001, 2020). These

differences between the populations may be influenced by variations in prey species, habitat characteristics and exposure to environmental pressures, as well as by underlying health issues.

4.3.2 Consequences of lung nematode infestations in the respiratory system

Lung nematodes can trigger a myriad of health complications within the respiratory system. Besides tissue calcifications by encapsulation or embedment within the lung parenchyma, they are also frequently associated with tissue damage and necrosis due to exceeding accumulation (Fig. 2B; Siebert et al., 2001, 2020; Ten Doeschate et al., 2017; Woodard et al., 1969; Zappulli et al., 2005). Furthermore, several studies have found a link between severe parasitic infestations and increased bacterial colonisation, promoting the contraction of secondary infections (Jauniaux et al., 2002; Jepson et al., 2000; Siebert et al., 2001). Exceeding nematode numbers and subsequent inflammation may impair the ability to eliminate bacteria, thereby facilitating viral or bacterial infection (Fig. 2B). Pneumonia is frequently observed alongside high lungworm burden, further impeding the health status of ailing individuals and is often reported as a leading cause of death in harbour porpoises (Jauniaux et al., 2002; Reckendorf et al., 2021; Siebert et al., 2006a, 2006b, 2009; Wünschmann et al., 2001). Lung nematodes are mobile within their host and have been observed in blood vessels, where they can obstruct blood flow and increase the risk of thrombosis, sclerosis, and hypertension (Fig. 2B; Carvallo & Stevenson, 2022; Ceco et al., 2017; Jepson et al., 2000; Siebert et al., 2006b; Ten Doeschate et al., 2017). Nematode-associated impacts on the respiratory system can have serious implications for harbour porpoises, as parasites are regularly detected in multiple organs of severely affected individuals which often suffer from multi-organ diseases (Jauniaux et al., 2002; van Elk et al., 2019). Consequently, high nematode burden may impact lung function and capacity, thus impair diving ability and affect overall host fitness (Fischbach & Seguel, 2023; Siebert et al., 2001).

As an interface of the animal with its environment and constant exposure to pathogens, the respiratory tract possesses various defence mechanisms to mitigate their impact (Beineke et al., 2010; Di Guardo et al., 2019; Haley, 2003). In response to alveolar damage and parasitic infestations, a T helper cell type 2 (Th2) activation is the typical reaction of the lung tissue (Allen & Maizels, 2011; Sandler et al., 2003; Tsang et al., 2025). Generally, the immune reaction can be broadly described by three phases (Ware & Matthay, 2000). During the acute phase, the lung tissue experiences damage and integrity is disrupted. Resident immune cells then trigger a proinflammatory response through cytokine activation (Keane, 2008; McClintock

et al., 2008), which recruits macrophages that degrade the extracellular matrix (ECM), enabling neutrophil infiltration to the site of damage (Wilson & Wynn, 2009). The organising phase is characterised by proliferating lung cells which are largely responsible for regeneration and reinstating tissue function (Wilson & Wynn, 2009). If tissue function cannot be regained and fibrosis and inflammation persist, the chronic-fibrotic phase is reached. The alveolar surface thickens, lung tissue can scar and irreversibly lose its function, so no sufficient respiration is feasible (Carvallo & Stevenson, 2022; Matute-Bello et al., 2008). Prolonged immune activation can also result in a negative feedback loop that exacerbates inflammation, tissue loss and subsequent respiratory distress (Tighe & Noble, 2011). While a thickened alveolar wall and increased elastic fibres is associated with pulmonary fibrosis in humans, this phenotype poses as an adaptation in cetaceans to enable alveolar collapse (Matute-Bello et al., 2008; Piscitelli et al., 2010, 2013). Likewise, a recent molecular study found an evolutionary loss of a plethora of pulmonary fibrosis-associated genes in whales which may further attenuate potential lung injuries (Guo et al., 2024). Interestingly, toothed whales possess a resident macrophage type, the pulmonary intravascular macrophage (PIM), that is reactive to lipopolysaccharides (LPS), cellular debris and other particles transported in from the vasculature (Carvallo & Stevenson, 2022; Kawashima et al., 2004; Schneberger et al., 2012). Macrophages have various functions, including attenuation of inflammation and degradation of debris and other organisms (Murray & Wynn, 2011). However, PIMs are thought to raise the susceptibility of lung injury in toothed whales by aggravating lung inflammation due to their exceeding immune signalling between the pulmonary endothelium and the vasculature (Kawashima et al., 2004; Schneberger et al., 2012).

4.3.3 Host-parasite dynamics, co-evolution and selective pressure

The dynamics between hosts and parasites influence each other and can exert selective pressures, as they shape host immunity, vulnerability and resilience (Vicente-Santos et al., 2023). This coevolutionary dynamic may proceed in a pattern, whereby as the host evolves defensive responses to the parasite, the parasite may in turn evolve strategies that increase virulence (Penczykowski et al., 2016). An example of such an evolutionary arms race is observed in parasitic nematodes which facilitate chronic infection through production of molecules (e.g., nucleic acids, secretory molecules) that manipulate the host immune system via immunomodulation or molecular mimicry (Harnett, 2014; Johnston et al., 2017). Parasites can also put selective pressure on populations by disproportionately affecting susceptible individuals. Young, elderly, diseased or malnourished individuals are more likely to succumb

to parasite infection, thereby influencing genetic diversity and population structure (Penczykowski et al., 2016; Vicente-Santos et al., 2023). As endoparasites, nematodes depend on their host for replication and survival, and are expected to favour a balanced host-parasite dynamic. A balanced dynamic promotes tolerance of parasites within the host, hence enables control of infection severity and prevents overactivation of the immune system (Penczykowski et al., 2016). A similar host-parasite dynamic may have evolved between lung nematodes and harbour porpoises, in which these parasites are frequently observed. Despite pulmonary infestations, individuals from the North and Baltic Seas appear to be in good body condition, which often serves as a health indicator. This finding has led to the hypothesis that harbour porpoises may be unaffected by the presence of lung nematodes and may have developed tolerance strategies (Kirkwood et al., 1997; van Elk et al., 2019). Likewise, harbour porpoises may possess a certain level of tolerance to mild to moderate lung nematode infestations (Tsang et al., 2025). To do so, they appear to manage parasitic load through a strong Th2 inflammation, similar to other animals in response to nematodes (Degen et al., 2005; Weatherhead et al., 2020). However, harbour porpoises in the North and Baltics Seas are exposed to a range of additional stressors that may cumulatively impede their ability to successfully mitigate the impacts of parasitic infestations (IJseldijk et al., 2022; Koschinski et al., 2024; Ten Doeschate et al., 2017; Vicente-Santos et al., 2023).

4.4 Anthropogenic activities and their impact on marine mammal health

The North and Baltic Seas have developed into two of the most intensively utilised marine regions (Halpern et al., 2008; Kaplan & Solomon, 2016). While the quantification and mitigation of the highly variable effects of natural and anthropogenic pressures on harbour porpoises remain complex (Simmonds, 2017; Simmonds et al., 2014), these pressures may exert cumulative effects and influence population-level health (Fig. 3; Carlén et al., 2021; Leal Filho et al., 2022; Nelms et al., 2021).

4.4.1 Underwater-radiated noise may impact harbour porpoises

Exposure to underwater-radiated noise has become one of the main concerns of marine mammal conservation and is the focus of a growing number of studies and mitigation efforts (Dähne et al., 2017; Dietz et al., 2021; Erbe et al., 2019; Richardson & Würsig, 1997; Southall et al., 2019; Wisniewska et al., 2018). As shipping, offshore construction, piling and seismic activities

increase, the acoustic pollution in today's oceans has risen incessantly, consequently affecting marine mammals (Fig. 3; Erbe et al., 2019; Gomez et al., 2016; Hildebrand, 2009; Southall et al., 2021; Weilgart, 2017). Many contemporary acoustic noises overlap with the hearing range and biosonar of harbour porpoises, hence impact their spatial orientation, foraging and communication (Dähne et al., 2013; Southall et al., 2021; Tougaard et al., 2015). Studies have observed a halt of foraging, cessation of communication, and temporary or permanent avoidance of the disturbed area, even if the noise source is detected from a distance (Akkaya Bas et al., 2017; Dähne et al., 2013; Dyndo et al., 2015; Frankish et al., 2023; Nehls et al., 2023; Oakley et al., 2017; Terhune & Kastelein, 2025; Wisniewska et al., 2018). Some individuals may also startle and remain unresponsive, which has been suggested to indicate an already stressed and potentially weakened animal unable to reinforce energy to flee from the source (Brennecke et al., 2022; Elmegaard et al., 2023; Southall et al., 2021; Voß et al., 2023). In addition, the use of seal deterrents, deployed to prevent depredation of fisheries by marine mammals, can provoke a rapid flight response or spontaneous, prolonged bottom diving, atypical for harbour porpoises, in an attempt to evade the noise source (Elmegaard et al., 2023; Schaffeld et al., 2019; Voß et al., 2023).

4.4.2 Pollution and reduced prey availability may weaken immune defences

Permanent displacement and loss of key habitat due to disturbance, together with shifts in prey abundance caused by overfishing, can increase inter- and intraspecies competition for limited resources such as food and mates (Laidre et al., 2008). These factors may be further aggravated by global climate change, as rising ocean temperatures can alter abiotic factors (e.g., salinity, acidity, oxygen saturation; Hoegh-Guldberg & Bruno, 2010), promote emerging infectious diseases and unusual mortality events, and shift species distribution and prey abundance (Broadwater et al., 2018; Burge et al., 2014; Evans et al., 2010; Evans & Bjørge, 2013; Heide-Jørgensen et al., 2011; Nelms et al., 2021; Pigeault et al., 2024; Santora et al., 2020; Sharpe & Berggren, 2024; Simmonds & Elliott, 2009; Simmonds & Isaac, 2007). Although harbour porpoises are generalist feeders and switch to more abundant prey when their preferred food sources decline, diet studies of harbour porpoises, harbour seals and grey seals have revealed overlaps in key prey species (Heße et al., 2025; Planque et al., 2021), potentially intensifying competition. Furthermore, even if prey stocks recover, harbour porpoises do not necessarily reintroduce these species into their diet, which could have adverse consequences if high-quality prey species are lost (Santos & Pierce, 2003; Stedt et al., 2024).



Figure 3 Cumulative anthropogenic impacts on marine mammals. Harbour porpoises are exposed to a plethora of human activities, both on land and at sea. Industrial and chemical waste can wash contaminants into the sea which bioaccumulate in marine mammals. Agrarian runoff can elevate nutrient concentrations that fuel harmful algae blooms. Rising ocean temperatures due to climate change may also contribute to algae blooms and additionally lead to displacement of marine mammals or key prey species. Fisheries can not only cause prey depletion, but are also the main reason for high numbers of entanglement. Discarded nets and rising ocean pollution further cause entanglements and can be confused for food. Another rising concern is underwater-radiated noise, which is exacerbated by increasing shipping, seismic surveys, naval exercises and offshore construction. The overall influence of noise can lead to habitat loss, reduced foraging and socialising, and hearing damage. Illustration by Eda Dönmez

Harbour porpoises have a high metabolic rate, thus have a higher risk of starvation (IJseldijk et al., 2022; Kastelein et al., 1997b, 2019; Wisniewska et al., 2018). To maintain a thick fat layer (blubber) for insulation and as energy reserve, they need to consume vast amounts of high-quality prey (Kastelein et al., 2019; Rojano-Donãte et al., 2018). Blubber thickness is often used as a proxy to estimate health (Kershaw et al., 2017). Harbour porpoises in densely trafficked areas of the North Sea possess substantially less thick blubber compared to harbour porpoises residing in less humanly frequented Scandinavian and Arctic waters (Kershaw et al., 2017; Siebert et al., 2006a, 2009; Wünschmann et al., 2001). These populations also appear to display less severe pathological lesions and inflammations in contrast to the North Sea population (Siebert et al., 2001, 2006a, 2009, 2020), suggesting a potential link between reduced blubber and vulnerability to disease and environmental pressures. However, blubber thickness does not

necessarily accurately reflect health condition. Despite exhibiting considerable lungworm load and associated pneumonia, harbour porpoises have previously been found to be in good nutritional state (Ryeng et al., 2022; Siebert et al., 2020). Body condition is also not representative of potential sub-lethal impacts, such as contaminant accumulation (Fig. 3). Despite restrictions or bans of chemicals (e.g., mercury) and long-lasting contaminants in Europe (e.g., persistent organic pollutants, POPs 2001; dichlorodiphenyltrichloroethane, DDT 1970s; perfluoroalkyl and polyfluoroalkyl substances, PFAS 2023, polychlorinated biphenyls, PCBs 1985, microplastic, 2023), they persist in the marine environment and bioaccumulate in harbour porpoises (Beineke et al., 2005; Siebert et al., 2009; Sonne et al., 2020b). As highly lipophilic contaminants, they are stored in the blubber and are released upon blubber metabolism during periods of low food availability (Beineke et al., 2005; Desforges et al., 2016; Kastelein et al., 2019; Koopman et al., 2002; Siebert et al., 2009; Sonne et al., 2020b; Weijs et al., 2022). Considering that malnourished animals can experience a higher burden and diversity of parasites and diseases, potentially due to inability to reinforce effective immune responses (Vicente-Santos et al., 2023), a sudden release of immunocompromising agents can significantly affect resilience and overall fitness. Contaminant accumulation has not only been associated with a compromised immune system (Desforges et al., 2016; Mahfouz et al., 2014; Reiter et al., 2022; Sonne et al., 2020b; Weijs et al., 2022), but also with reduced reproduction success in harbour porpoises (Murphy et al., 2015; Reijnders, 1986; Wells et al., 2005; Williams et al., 2021). Concerningly, the offspring is almost immediately exposed to contaminants, as mothers offload up to half their contaminant burden to their calves (van den Heuvel-Greve et al., 2021). Because the immune system of young individuals may not be fully developed, they may be inadequately equipped to respond to such challenges, increasing mortality risk prior to reproductive age and contributing to a potential long-term population decline.

4.5 Oxygen distribution in cetaceans: the lungs, blood, and skeletal muscles

4.5.1 The primary site of oxygen uptake: the lungs

The respiratory tract is the first site of gas exchange and management, so understanding the adaptations of the cetacean respiratory tract and lung is essential to elucidate how these animals can perform their diving bouts (Piscitelli et al., 2010; Reidenberg & Laitman, 2025).

Whales have developed remarkable physiological adaptations that enable their diving ability despite periodical access to oxygen (Davis, 2014). One of the most prominent adaptations is the lung collapse at depth while diving, which reduces their gas exchange and diminishes the risks of related injuries, such as nitrogen narcosis or decompression sickness (Fahlman et al., 2017, 2020, 2021; Hooker et al., 2012; Ridgway & Howard, 1979). The depth at which lung collapse occurs is not homogenously timed, but heterogeneously to supply air to low-oxygen tissues, while reducing nitrogen saturation levels (Fahlman et al., 2021). Although lung collapse is more profound in deep-diving whale species, computed tomography lung measurements of post-mortem harbour porpoises have indicated that they are capable of lung compression and collapse (estimated at 133 m; Moore et al., 2011). Alveolar collapse and re-inflation are anatomically strengthened by abundant elastic fibres (myoelastic sphincters) and thickened alveolar walls that enable constriction of the terminal airways and control of air flow distribution (pulmonary shunt; Reidenberg & Laitman, 2025), though the exact mechanisms still remain unknown (Fanning & Harrison, 1974; Kooyman & Cornell, 1981; Kooyman & Sinnett, 1979; Piscitelli et al., 2010, 2013; Ponganis, 2011). This may also prevent mechanical stress during ascent and descent, and rupture during ventilation upon re-emergence (Fanning & Harrison, 1974; Piscitelli et al., 2013). The alveolar re-opening during ascent is supported by surfactant production by the alveolar epithelium which lowers the risk for surface hypertension in the lung and boosts gas exchange (Foot et al., 2006). Upon re-emergence at the sea surface, harbour porpoises perform a single exhalation and explosively exchange their complete gas volume (< 1 s; Kooyman, 1973; Kooyman & Sinnett, 1979; Reed et al., 2000). Moreover, the rather large alveolar surface area of the petite harbour porpoise enables a greater gas exchange ability, such as of oxygen (Fahlman, 2024; Reidenberg & Laitman, 2025; Schulze, 1908). As they are generally less affected by pressure-related injuries, shallow-diving cetaceans also utilise their lungs as an oxygen reservoir (Fahlman et al., 2017; Piscitelli et al., 2010, 2013; Snyder, 1983).

Oxygen is efficiently extracted from the lungs via the bloodstream. In harbour porpoises and other cetaceans, this process may be enhanced by higher blood oxygen affinity and elevated oxygen-transporting haemoglobin concentrations compared to terrestrial mammals (Kooyman et al., 1980b; Soegaard et al., 2012). Furthermore, harbour porpoises may induce vasodilation of the pulmonary vasculature via nitric oxide during dives, thereby maintaining pulmonary blood flow (Soegaard et al., 2012). Oxygen transported in the blood is subsequently bound by myoglobin in the skeletal musculature. Myoglobin is highly abundant in cetacean muscle tissue

and also exhibits high oxygen affinity, functioning as an effective oxygen storage that can be readily mobilised during dives (Arregui et al., 2021; Castellini et al., 1981).

4.5.2 The demanding end-organ: the skeletal muscles

The skeletal muscles are essential for propulsion and diving bouts in cetaceans, therefore consume vast amounts of energy and oxygen (Arregui et al., 2021; Pabst, 1993). To meet these demands, whales possess not only high myoglobin concentrations for oxygen storage, but also high concentrations of intramuscular fat and glycogen droplets that serve as energy reserves during dives (Dannenberger et al., 2020; Tulsi, 1975). Most marine mammals primarily dive aerobically and rarely exceed their aerobic dive limit (ADL), which is defined by the dive duration after which blood lactate content exceeds resting concentrations (Butler & Jones, 1997; Kooyman, 1973; Kooyman & Ponganis, 1998; Martin & Smith, 1999; Towers et al., 2019). When diving beyond this limit, they rely on anaerobic metabolism, leading to tissue accumulation of lactate (Arregui et al., 2021; Castellini et al., 1981; Kooyman et al., 1980a; Torres-Velarde et al., 2021). Lactate is generated as a by-product of glycolysis and high concentrations can be cytotoxic (Lee et al., 2015). Whales therefore need to clear accumulated lactate through extended respiration intervals and by staying within shallow depths after resurfacing (Castellini et al., 1992). Although harbour porpoises are classified as shallow and short divers, in exceptional stress situations or upon finding promising prey patches, they can perform deeper dives that may exceed their ADL (~5 min; Reed et al., 2000), hence rely temporarily on anaerobic energy. However, muscles of harbour porpoises develop a high anaerobic buffering capacity early in life which may enhance anaerobic tolerance (Noren, 2004, 2014).

Additionally, periodical access to oxygen during diving bouts generates high levels of reactive oxygen species (ROS) upon tissue reoxygenation (Vázquez-Medina et al., 2006). ROS can form naturally during metabolic processes and as signalling molecule or during stress conditions (e.g., hypoxia; Sies & Jones, 2020). ROS are highly reactive and can interfere with DNA synthesis, induce inflammation, and cause cell damage. To mitigate oxidative stress and tissue damage, marine mammal muscles possess high baseline concentrations of antioxidants that bind and detoxify ROS (Allen & Vázquez-Medina, 2019; García-Castañeda et al., 2017; Vázquez-Medina et al., 2006). The positive selection and accelerated evolution of several antioxidant-related genes in whales and other marine mammals further underscores the importance of this protective adaptation (Foote et al., 2015; Li et al., 2021; Park et al., 2015; Romano et al., 2002).

As the skeletal muscle can accumulate oxidative damage, increasing proteolysis and ultimately leading to muscle wasting, adequate muscular regeneration is crucial to maintain tissue function (Frontera & Ochala, 2015; Howard et al., 2020; Lian et al., 2022; Rom & Reznick, 2016). Generally, the regeneration process follows a highly conserved sequence. Firstly, impacted cells and tissue areas are degraded and then disposed of to avoid cytotoxic accumulation of cell debris (Grounds, 2014; Levine & Kroemer, 2019). This is followed by inflammatory processes of the area by recruiting immune cells and completed by rapid regeneration and remodelling of the affected area to ensure continued tissue function (Grounds, 2014; Levine & Kroemer, 2019). Healthy muscles are essential to enable diving in cetaceans. In accordance with this, muscles of fasting seals indicated a metabolic preference that shifted towards fatty acid metabolism, instead of muscle catabolism, potentially preserving muscle function (Wright et al., 2020). In beluga whales, overall body condition has been linked to muscle oxygen storage capacity (Choy et al., 2019), which may have implications for diving ability. This connection is plausible, as poor body condition often is associated with compromised health and nutrition states, with muscle tissue serving as a final energy reserve (Frontera & Ochala, 2015; Wolfe, 2006), thus may have adverse effects on muscle function and diving.

4.5.3 Implications of reduced oxygen for a diving mammal

For diving mammals, impaired respiratory function due to lung nematode infestation and associated diseases can be particularly costly. Because these species rely on optimal respiratory capacity to dive and forage, even minimal alterations may result in severe consequences, including multi-organ dysfunction, reduced diving ability and ultimately, decreased survival. Indicators that lung function is considerably impaired in cetaceans suffering from parasites and diseases have been observed in studies of harbour porpoises and other cetacean species. In dolphins, lung disease has been found to influence altered lung compression, thus likely exert an effect on dive behaviour and endurance (Moore et al., 2011). Pneumonia in bottlenose dolphins can reduce pulmonary airflow by 40% (Borque-Espinosa et al., 2020) and similarly, a harbour porpoise with a larger lung abscess displayed a reduction in exchanged lung gas volume per breath compared to healthy individuals (mean: 0.6 l; mean healthy: 1.2-2.2 l for older individuals, 0.2-0.9 l for younger individuals; Kastelein et al., 1997a). Also, observations in harbour porpoises in human care suggest that they may not utilise their complete lung capacity (approximately 60%; Rojano-Donãte et al., 2018), although this may not directly apply to free-ranging individuals. While it is difficult to assess whether cetaceans with parasitic infestations experience a significant reduction in oxygen uptake due to a compromised lung function that

progresses to comorbidities, studies in humans and model organisms have demonstrated that lung disease can negatively affect the skeletal muscle. Globally prevalent lung diseases, such as bronchopneumonia, chronic obstructive pulmonary disease (COPD), and pulmonary fibrosis, result in significantly impaired lung function and oxygen uptake, and have been shown to alter muscle fibre composition, reduce muscle function and accelerate muscle tissue loss (Ceco et al., 2017; Gea et al., 2013; Jaitovich & Barreiro, 2018). Declining lung health in cetaceans may therefore cause similar whole-body consequences. However, cetaceans likely face higher costs and trade-offs, as optimal lung function and oxygen supply to the skeletal muscles is critical to maintain diving ability and survival.

4.6 Omics in marine mammal science

Omics studies in marine mammal science are an emerging field that has been growing constantly due to the richness of insights one can gain, even with little amount of sample (Collí-Dulá & Ruiz-Hernández, 2022; Kebke et al., 2022; Mancía, 2018; Van Cise et al., 2024; Veldhoen et al., 2012). The access to samples is also the main limiting factor for marine mammals, as many are endangered and fall within strict protective conservation measures (Van Cise et al., 2024). Additionally, sampling remains challenging due to their aquatic lifestyle, which is why most current omics studies focus on material collected during health assessments or from biopsies, e.g., skin, blubber and blood (Mancía, 2018; Van Cise et al., 2024). As a result, reference data is still insufficient and decreasing across levels, from transcriptomics to metabolomics, not only in terms of published studies but also in terms of method sensitivity (Mancía, 2018). However, technological advances in the “omics era” and increasing data, such as the recently published harbour porpoise reference genome (Autenrieth et al., 2018; Davison et al., 2025) or blow proteome description of healthy dolphins (Bergfelt et al., 2018), foster more in-depth research into cetacean biology and adaptations. Additionally, combining multiple omics datasets into one analysis can give insights into the complex dynamics of systems biology, hence elucidate phenotypic changes due to changes of the environment or condition (Collí-Dulá & Ruiz-Hernández, 2022). Furthermore, these integrative approaches are emerging as tools to identify molecules that differentiate between disease states or environmental pressures, supporting the development of biomarker panels for cetacean health monitoring.

4.6.1 Transcriptomics and differential gene regulation

Transcriptomics, which analyse the gene expression for a distinct timepoint or condition, e.g., time of death, or before and after a stressor exposure, is one of the more established omics in the marine mammal field. One method to generate transcriptomes is RNA-Sequencing, a high-throughput sequencing method that quantifies the entirety of RNA (Stark et al., 2019; Wang et al., 2009).

Transcriptomic studies in whales and dolphins have been conducted for various species and tissues under many environmental or physiological conditions. Studies of the blood transcriptome of dolphins analysed the gene expression patterns to handling stress (capture-release) which initiates an acute stress response as evidenced by an increased hypothalamic-pituitary-adrenal axis (Mancia et al., 2008). Similarly, blood, blubber and skin transcriptomes have been used to examine toxicological reactions to contaminants in cetaceans (Bjørneset et al., 2023; Panti et al., 2011; Righetti et al., 2014; Simond et al., 2019, 2025; Trego et al., 2019a, 2019b). Following an oil spill, the blood transcriptomes of dolphins revealed an elevation of Th2 immunity, alongside expression patterns indicative of mitochondrial dysfunction and declines in cardiopulmonary and reproductive function over several years (Morey et al., 2022). In seals and seal cell cultures under stress, transcriptomic analyses of blubber and muscle tissues identified dysregulated metabolic pathways and differentially expressed genes involved in tissue remodelling, immune responses and cell cycle regulation (induced stress via ACTH, Deyarmin et al., 2020; blubber cell culture under stress hormone exposure, Kashiwabara et al., 2023; fasting, Martinez et al., 2018; seal cells under glucocorticoid stress, Torres-Velarde et al., 2021). Furthermore, transcriptomics have been used to investigate the potential adaptations behind the remarkable diving capacity and hypoxia tolerance of cetaceans (Geßner et al., 2022; Krüger et al., 2020).

4.6.2 Proteomics and insights into protein translation

The proteome gives insights into the protein composition in a tissue or cell type at a distinct timepoint and can reflect the phenotypic response to a stimulus. To quantify the abundance of present proteins, various methods exist. In contrast to traditional protein quantification methods that target one specific protein, such as Western Blots, untargeted approaches utilising liquid chromatography-tandem mass spectrometry (LC-MS/MS) allow the detection of a myriad of proteins.

Proteome studies of whales are a comparatively recent development. They have been used primarily in an ecotoxicological context, and for description of potential health biomarkers as proteins are constantly modulated to external or internal stimuli (Deyarmin et al., 2020; Kershaw et al., 2024). Blubber proteomes of wild harbour porpoises and minke whales revealed an abundance of proteins involved in lipid metabolism, and immune system and inflammation (Kershaw et al., 2018, 2024). Similarly, the proteome of blow condensate from bottlenose dolphins has been analysed in regard to health status and as diagnostic tool (Bergfelt et al., 2018), providing a basis for non-invasive monitoring.

4.6.3 Metabolomics and lipidomics and abundance of intermediate molecules

Metabolomic and lipidomic studies remain relatively underexplored as an omics method applied to marine mammals. These methods can be utilised to quantify altered macromolecule compositions (e.g., carbohydrates, proteins and lipids), as a consequence of ecotoxicological impacts or physiological adaptations (Senevirathna & Asakawa, 2021). While fluctuations in prey source can alter measured metabolites and lipids (Bories et al., 2021; Monteiro et al., 2021), bioactive metabolites and lipids regulate pathways, and represent the end-product or intermediate molecules of altered gene and protein expression (Chiurchiù et al., 2018; Hannun & Obeid, 2008).

Metabolomic studies in cetaceans have been used especially for blow exhale analyses, a completely non-invasive sampling method (Aksenov et al., 2014; Borrás et al., 2017; Harsla et al., 2024; Zamuruyev et al., 2016), and to assess metabolic disorders and health conditions (Pasamontes et al., 2017; Ruiz-Hernández et al., 2022; Weijs et al., 2022). Stress-induced metabolite changes have also been observed in marine mammals, including the highly contaminant-impacted St. Lawrence beluga population, which endure similar anthropogenic impacts like the harbour porpoises inhabiting the North and Baltic Seas (Champagne et al., 2013; de Lima et al., 2024; Ruiz-Hernández et al., 2022; Simond et al., 2020, 2022, 2025). Moreover, a study using untargeted plasma metabolomics compared dolphins exhibiting varying health states and found constraints in amino acid metabolism and increased protein degradation in individuals with poor health (Derosus et al., 2022).

4.7 Aims and objectives of this project

Harbour porpoises act as top predators in German waters, thus can also be indicative of changes within the ecosystem. With anthropogenic activities rising in the North and Baltic Seas, their populations are negatively affected and long-term monitoring efforts, including post-mortem investigations, have observed declining health and fitness. In particular, harbour porpoises are frequently diagnosed with pathologies of the respiratory system, in which nematode infestations are predominantly observed. A highly impacted respiratory tract is also a concern in regard to sufficient oxygen uptake and supply to consuming end-organs, such as the brain, heart and skeletal muscle. Heavy accumulations of nematodes, in addition to secondary diseases, may ultimately negatively affect the diving ability of harbour porpoises, as well as their foraging success. The aim of this project was to analyse whether harbour porpoises developed molecular mechanisms which may aid in compensation of negative effects on pulmonary function, and oxygen uptake and supply caused by severe nematode infestations. As lung nematodes also put selective pressure on their host, harbour porpoises may possess mechanisms that enable them to tolerate certain levels of infestations, hence remain able to forage and stay competitive. Due to their fully aquatic lifestyle, shy nature and protection status, investigations in free-ranging harbour porpoises are challenging. Here, lung and skeletal muscle tissues were collected from stranded or by-caught harbour porpoises that were subjected to routinely performed post-mortem examinations. Utilising high-throughput methods, including transcriptomics, proteomics, metabolomics and lipidomics, in combination with state-of-art, molecular tools, the presented studies enable deeper insights into the pathophysiology of severe lung nematode burden and potential systemic effects and responses in harbour porpoises. Lastly, this project aimed to identify and propose a potentially suitable biomarker panel to support peripheral health assessment of harbour porpoises and population monitoring.

5 Chapter I

Diving on damage — the muscle transcriptome of parasitic infested harbor porpoises (*Phocoena phocoena*) hints at oxidative stress but not hypoxia

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Abstract

The only native cetacean in German waters, the harbor porpoise (*Phocoena phocoena*), is impacted by numerous pathological lesions in the respiratory tract mainly caused by parasites or bacteria. Although harbor porpoises have been observed to not use their complete lung volume, it has not been studied whether this insufficiency leads to lower oxygen uptake, impaired diving ability, and, ultimately, reduced foraging success. This project aims to analyze whether harbor porpoises developed novel molecular adaptations to compensate impairments in oxygen supply, thus remaining viable and competitive despite the high parasitic load. Here, initial comparative transcriptome RNA sequencing (NextSeq 2000, Illumina) was performed on muscles of harbor porpoises with a respiratory tract considered as healthy and of harbor porpoises that suffered from more severe lesions and parasitic infestations in the respiratory tract. Our findings suggest an elevated response to oxidative stress in the muscles of parasitic infested harbor porpoises compared with that of healthy animals. Higher antioxidant and antiapoptotic gene expression in the muscles of non-healthy harbor porpoises might function as a compensatory effect to enhanced reactive oxygen species production and accumulation in the muscles. Simultaneously enhanced selective proteasomal degradation and myogenesis suggest a tightly controlled, finely tuned switch of the intrinsic muscle response to stress. Lipid metabolism pathways and rate-limiting transcripts involved in glycolysis were upregulated and may uphold muscle energy supply for tissue function and energy-consuming regenerative and biosynthetic processes. These preliminary results hint at a defined response of the muscle to oxidative stress that may be caused by lung tissue with more severe pathological lesions and may indicate a possible adaptation in cetaceans.

KEYWORDS cetacea, hypoxia, oxidative stress, muscle, transcriptome, harbor porpoise, marine mammals

Introduction

In the North and Baltic Seas, harbor porpoises (*Phocoena phocoena*) are increasingly impacted by ever-expanding human activities, leading to an endangered status (Carlén et al., 2021; Nachtsheim et al., 2021). Although disturbances by shipping and underwater noise during feeding and foraging are especially detrimental due to their high metabolic rate and restricted

energy storage capacity (Read & Hohn, 1995; Rojano-Don te et al., 2018; Wisniewska et al., 2016, 2018), it has been observed that underwater noise even leads to behavioral changes (Kastelein et al., 2018). Moreover, not only is entanglement in fishing gear and bycatch affecting population sizes, but growing contaminant exposure is thought to reduce reproduction success in female harbor porpoises (Kesselring et al., 2017; Vinther & Larsen, 2004). Accumulating exposure to chemical waste and pollution as well as microplastic can compromise the immune system of the harbor porpoises (Philipp et al., 2021; Sonne et al., 2020a, 2020b). Uptake through contaminated food sources allows for accumulation of long-lasting pollutants in sentinel species such as the harbor porpoise (Siebert et al., 2009; Sonne et al., 2020b). This can weaken the ability of the immune system to defend against illnesses and infections, thus leading to a higher susceptibility to more frequent and severe infections or infestations with parasites (Siebert et al., 2009, 2020; W nschmann et al., 2001). Compared with Scandinavian and Arctic populations, German harbor porpoises possess less blubber while also suffering from severe pathological lesions and inflammations (Siebert et al., 2006a, 2009; W nschmann et al., 2001). Particularly in the respiratory tract and lungs, considerable injuries have been determined, which are mainly caused by parasites or bacteria (Lehnert et al., 2014; Siebert et al., 2001, 2020). It has been proposed that large accumulations of parasites in the respiratory tract may negatively affect the diving and hunting ability of the harbor porpoises (Siebert et al., 2001; Ten Doeschate et al., 2017).

To effectively dive and hunt under water, marine mammals possess a plethora of adaptations that enable them to live in an aquatic environment (Hindle, 2020). They prefer to dive aerobically and possess a large amount of readily available oxygen stored in their blood, lungs, and muscles (Davis, 2014; Kooyman, 1973). The locomotor muscles of marine mammals consume vast amounts of oxygen during aerobic dives to produce propulsion and force (Pabst, 1993). A heterogenic distribution of oxygen-binding myoglobin and higher concentrations in muscles of deep-diving species has already been confirmed, strengthening the suggestion that high myoglobin concentrations in oxidative, high-energy tissues prolong the aerobic dive limit (Polasek & Davis, 2001). Whereas deep-diving species preferably use the muscle-stored oxygen, shallow-diving whales such as the harbor porpoise also rely on their lungs as an oxygen storage (Fahlman et al., 2017; Snyder, 1983). Although their dives are typically slow, short, and shallow (14–32 m; Nielsen et al., 2018; Otani et al., 2001; Piscitelli et al., 2010; Westgate et al., 1995), they can perform deeper foraging dives (226 m; Nielsen et al., 2018; Westgate et al., 1995). Moreover, porpoises of the Kattegat and Belt Sea have been observed trying to escape underwater noise with unplanned, deeper dives (Wisniewska et al., 2018). These

prolonged dives of marine mammals are typically fueled by glycolysis following depletion of oxygen storages (Arregui et al., 2021; Castellini et al., 1981; Kooyman et al., 1980a; Torres-Velarde et al., 2021). For small marine mammals like the harbor porpoise, it has been proposed that a high anaerobic buffering capacity of the locomotor muscles may extend their ability to dive aerobically (Noren, 2004). This could be true for fleeing or distressed harbor porpoises that, at birth, exhibit already 69% of the adult muscle anaerobic buffering capacity (Noren et al., 2014). In marine mammals, high baseline antioxidant capacity and levels have been confirmed in various tissues and species to protect from oxidative damage (Allen & Vázquez-Medina, 2019; García-Castañeda et al., 2017; Vázquez-Medina et al., 2006). Furthermore, growing evidence supports accelerated evolution or positive selection of antioxidative genes in marine mammals (Foote et al., 2015; Li et al., 2021; Park et al., 2015; Romano et al., 2002).

A recent study in belugas found that muscle oxygen storage capacity is correlated with overall body condition (Choy et al., 2019). However, the skeletal muscle is known to be a plastic tissue, swiftly adapting to changing conditions (Frontera & Ochala, 2015) with an elevated recovery capacity upon tissue injury or disease (Howard et al., 2020). Regeneration of muscles consists of a conserved three step response: degradation of affected cells, inflammation of the injured area, followed by tissue regeneration and remodeling (Grounds, 2014; Levine & Kroemer, 2019). Oxidative stress and hypoxia are known to induce muscle atrophy by increasing proteolysis and inhibiting translation of proteins (Lian et al., 2022). As a post-mitotic tissue, it is very prone to oxidative damage induced by reactive oxygen species (ROS) and can accumulate damage over time (Frontera & Ochala, 2015; Rom & Reznick, 2016). Multiple muscle transcriptome studies of stressed elephant seals have been published (Crocker et al., 2016; Hindle et al., 2019; Khudyakov et al., 2015a, 2015b; Piotrowski et al., 2021; Torres-Velarde et al., 2021), and a specific response pattern with minimal catabolism and tissue function and suppressed energy-consuming processes such as proliferation and development has been observed (Khudyakov et al., 2015a). To date, mostly histological, pathologies describing or physiological studies of harbor porpoises muscles exist (Lehnert et al., 2014; McDonald et al., 2018; Noren, 2004, 2014; Noren & Williams, 2000; Siebert et al., 2001; Sierra et al., 2013, 2017). This is the first transcriptomic study of the muscles of harbor porpoises.

Here, we have performed comparative muscle transcriptome analyses to investigate whether harbor porpoises developed molecular compensatory adaptations caused by damaged lungs and impaired lung function, thus remaining viable and competitive. We compared transcriptomes of the main locomotor muscle (*Musculus longissimus dorsalis*) from German harbor porpoises,

with a respiratory tract regarded as either healthy or non-healthy. *M. longissimus dorsalis* is one of the most energy-reliant muscles in cetaceans and necessary for the upward stroke of the fluke (Noren & Williams, 2000; Pabst, 1993), therefore representing an appropriate target to analyze possible adaptations in the muscle to an impaired respiratory function. We conducted Gene Ontology (GO) term analyses to find indicators of possible adaptations in non-healthy harbor porpoises. Furthermore, we identified differentially expressed transcripts (DETs) in non-healthy harbor porpoises compared with that in healthy ones and verified selected upregulated candidate genes in a quantitative real-time (qRT)–PCR.

Material and methods

Animals and sampling

M. longissimus dorsalis samples of harbor porpoises (n = 14, Table 1; see Supplementary data) were opportunistically obtained. All harbor porpoises died of live stranding or have been by-caught between 2015 and 2022. Fresh tissue samples were collected by the necropsy team of the Institute of Terrestrial and Aquatic Wildlife Research (ITAW), University of Veterinary Medicine Hannover, Foundation, Büsum. Tissue samples were immediately preserved in RNA stabilization solution (NucleoProtect RNA, Macherey-Nagel, Düren, Germany) and stored at –80 °C until subsequent usage. Full necropsies and further investigations were conducted on all individuals according to Siebert et al., 2001. On the basis of the summary of findings, animals were categorized into healthy and non-healthy individuals. Non-healthy animals displayed pathological lesions due to lungworm and bacterial infections and suffered or died from bronchopneumonia (Table 1; see Supplementary data).

RNA isolation and quality control

The muscle samples (20–30 mg) were minced and homogenized by bead beating in 1 mL of Trifast reagent (PEQLAB, Erlangen, Germany). RNA was isolated from the homogenates by phase extraction using chloroform and ethanol (70%). Total RNA was extracted with the Crystal RNA Mini Kit (Biolab Products, Bebensee, Germany) in accordance with the manufacturer's instructions. In addition, a 15-min on-column DNA I digest (Qiagen, Hilden, Germany) was conducted. RNA concentration and quality, indicated by the RNA integrity number (RIN), were assessed using the Agilent TapeStation System (Agilent Technology, Santa Clara, CA, USA). RIN scores of the muscle samples varied from 4.8 to 6.1.

Table 1 Important data of the individual harbor porpoise samples and for which experiments samples were used.

Pph	Used in	Condition	Sex	Date	Age (approx.)	Cause of illness	Cause of death
24876	qRT-PCR and RNA-Seq	Healthy	Male	19.08.2020	Neonate	None	Suspicion of bycatch
26079	qRT-PCR and RNA-Seq	Healthy	Male	23.11.2021	Juvenile	Age-appropriate minor inflammations in the lungs, stomach, and liver	Bycatch
21708	qRT-PCR	Healthy	Female	19.07.2016	Neonate	None	Bycatch
21805	qRT-PCR	Healthy	Female	15.09.2016	Juvenile	None	Bycatch
25159	qRT-PCR	Healthy	Male	19.11.2020	Juvenile	None, only signs of a shock event	Suspicion of bycatch
24936	qRT-PCR	Healthy	Male	03.09.2020	Neonate		Perinatal death (amniotic fluid aspiration, hepatic and renal fatty degeneration)
24548	qRT-PCR and RNA-Seq	Non-healthy	Male	12.03.2020	Juvenile		Bronchopneumonia and dermatitis
23771	qRT-PCR and RNA-Seq	Non-healthy	Male	16.04.2019	Juvenile		Bronchopneumonia and gastroenteritis
24394	qRT-PCR	Non-healthy	Male	18.12.2019	Adult		Bronchopneumonia and gastritis
24138	qRT-PCR	Non-healthy	Female	10.09.2019	Adult	Bronchopneumonia, hepatitis, and adrenalitis with final septicemia due to <i>Pasteurella multocida</i>	Suspicion of bycatch/trauma
25266	qRT-PCR	Non-healthy	Male	15.12.2020	Juvenile	Bronchopneumonia and steatitis	Suspicion of bycatch
25660	qRT-PCR	Non-healthy	Female	14.07.2021	Adult	Bronchopneumonia and endoparasitosis	Bycatch
26076	qRT-PCR	Non-healthy	Male	23.11.2021	Juvenile	Bronchopneumonia and gastritis	Bycatch
26174	qRT-PCR	Non-healthy	Male	08.02.2022	Juvenile		

For additional data of the samples, see Supplementary Data.

Sequencing and quality trimming

The RNA sequencing (RNA-Seq) library preparation for paired-end sequencing of 2×150 nucleotides (nt) was generated from 5,000 ng of RNA of healthy ($n = 2$) and non-healthy ($n = 2$) harbor porpoises. Sequencing was performed on an Illumina NextSeq 2000 platform (StarSEQ, Mainz, Germany) with an output of 25 million reads per sample. Sequence quality control, mapping, and alignment were carried out on the Galaxy server (version 21.09) of the

Department of Biology at the University of Hamburg. Quality control was assessed using the FastQC version 0.73 and MultiQC version 1.11 tool on Galaxy. The first 20 5'-terminal nucleotides and Illumina adapter sequences (mismatch count = 2, internal match = 10) were cropped from the raw reads with Trimmomatic version 0.38.1. Reads below the length of 20 nt were discarded, and a minimum average quality value of 20 was required for consideration.

The raw sequence files are available at the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) from (SRA BioProject ID: PRJNA977857, see Supplementary Table 1 for SRA accession numbers).

Expression analysis *via* RNA-Seq

The trimmed sequences were mapped and aligned against the bottlenose dolphin genome (*Tursiops truncatus*, mTurTru1.mat.Y, released March 2020, RefSeq Accession: GCF_011762595.1) using HISAT version 2.2.1 and featurecounts version 1.6.4. Individual transcripts per million (TPM) for each transcript were counted with the Galaxy tool “Generate CPM, TPM, RPK” (version 0.4.0), and mean values for the healthy and the non-healthy animals were calculated.

Differential expression analysis

DETs were determined using DESeq2 version 2.11.40.7 on the Galaxy server. DETs were then filtered with cutoffs for up- and downregulated genes at an adjusted p-value (p_{adj}) ≤ 0.05 , fold change (FC_{log2}) ≥ 1 or ≤ -1 , and mean $TPM_{non-healthy} \geq 5$ (see Supplementary data).

Gene Ontology analysis

Analysis of GO Slim terms was performed with the PANTHER Overrepresentation Test (PANTHER version 17.0, released 2022_02) using the human as reference list. DETs with a $p_{adj} \leq 0.05$, a $FC_{log2} \geq 1$ or ≤ -1 , and a mean $TPM_{non-healthy} \geq 5$ were considered for the analysis. Overrepresentation was determined in the PANTHER “Pathways” and GO Slim categories “Biological Process”, “Molecular Function”, and “Cellular Component” categories. Fisher’s exact test and the False discovery rate (FDR) were used as correction for multiple testing.

Expression confirmation *via* qRT-PCR

Because of our small muscle sample size for the RNA-Seq, we verified the results in a larger subset of healthy (n = 6) and non-healthy (n = 8) harbor porpoises (Table 1). mRNA expressions

of the selected transcripts chosen as candidate genes were analyzed in a qRT-PCR. Total RNA was isolated as previously stated. RNA quantity and quality were determined by spectrophotometry and gel electrophoresis. For complementary DNA (cDNA) synthesis, a maximum of 1,000 ng of total RNA and the RevertAid H⁻First Strand cDNA Synthesis Kit (Thermo Scientific, Germany) were used according to the manufacturer's protocol. The synthesized cDNA was further diluted in 20 µl of Ribonuclease (RNase)-free water, resulting in a total volume of 40 µl. qRT-PCR was conducted on the ABI 7500 real-time PCR system with the Power SYBR Green master mix (Applied Biosystems, Darmstadt, Germany). The qRT-PCR was performed with a protocol consisting of 40 cycles (95 °C for 15 s, 58 °C–60 °C for 60 s, 72 °C for 30 s). Species-specific primers are presented in Supplementary Table 2. Samples were applied as three technical replications. The qRT-PCR was used as a relative verification of the transcriptome analysis, so no recombinant plasmid was necessary as standard. RPLP0, RPLP1, and EF2 were selected as reference genes. Relative FCs were calculated according to the DDC_T method (Livak & Schmittgen, 2001).

Statistical analysis

All statistical analyses of the results qRT-PCR were conducted using GraphPad Prism version 9.5.1. Mean C_T values of the technical replicates were calculated and tested for normal distribution. The Mann–Whitney U-test was performed for *post-hoc* analysis and testing for statistical significance.

Results

Transcriptomes of the muscles

Per sample of non-healthy harbor porpoises, a total of ~43 million paired-end Illumina reads could be generated, and a total of ~25 million and ~36 million paired-end Illumina reads were generated per muscle sample of healthy harbor porpoises. The quality of the raw reads was sufficient (Phred score = 33). The mapping of the transcriptomes resulted in ~15 million ± 3 million mapped reads in healthy porpoises and ~21 million reads in non-healthy porpoises (67 ± 6%; Table 2). Alignment of the mapped reads against the bottlenose dolphin genome resulted in ~38.05 ± 3.45% assigned reads (Table 2). The analysis of DETs resulted in 1,594 upregulated and 1,286 downregulated DETs in muscles of non-healthy porpoises.

For further analyses, only DETs with a $p\text{-value}_{\text{adj}} \leq 0.05$, $\text{FC}_{\log 2} \geq 1$ or -1 , and a $\text{TPM}_{\text{non-healthy}} \geq 5$ were considered, comprising 739 upregulated transcripts and 194 downregulated transcripts (see Supplementary data).

Table 2 Mapping and alignment of the individual harbor porpoise transcriptomes.

Pph	Mapped (percent)	Aligned (percent)	Aligned (million reads)
23771	72.94%	41.20%	10.2
24548	71.44%	41.50%	9
24876	63.16%	36.30%	5.3
26079	60.60%	34.60%	7

Mapping and alignment are shown as percentage of the reference genome (Bottlenose dolphin) and the alignment additionally in million reads

High expressed transcripts in GO Slim terms for muscle function, regeneration, and glucose metabolism

We performed an overrepresentation analysis using the PANTHER categories GO Slim “Biological Process”, “Molecular Function”, “Cellular Component”, and “Pathways” to identify overrepresented GO Slim terms. For this, we only considered transcripts of the filtered set with cutoffs $\text{FC}_{\log 2} \geq 1$, $p\text{-value}_{\text{FDR}} \leq 0.05$, and $\text{TPM}_{\text{non-healthy}} \geq 5$ (see Supplementary data) with the human genome as reference (Fisher’s exact test with FDR-corrected $p\text{-value} < 0.05$). The top 10 overrepresented biological processes in muscles of non-healthy harbor porpoises compared with that of healthy porpoises are shown here (Figure 1). Many of the GO Slim terms were associated with skeletal muscle function and development (regulation of epithelial cell differentiation, Fold Enrichment (FE) = 18.38; myofibril assembly, FE = 8.36; muscle tissue development, FE = 7.29) as well as metabolic processes (glucose homeostasis, FE = 12.77; alpha-amino acid biosynthetic process, FE = 5.80; triglyceride metabolic process, FE = 6.13; fatty acid catabolic process, FE = 5.25). In addition, ribosome-related processes were found enriched (maturation of small subunit ribosomal RNA (SSU-rRNA) from tricistronic rRNA transcript, FE = 6.57; ribosomal large subunit biogenesis, FE = 7.07), and processes associated with cell death and clearance were enriched in the set of transcripts (autophagy of mitochondrion, FE = 6.57). The only identified, enriched GO Slim terms in the “Molecular Function” category were involved in “catalytic activity” and binding (snoRNA binding, transcription factor binding; Figure 1). Furthermore, we sorted the transcripts by their FC ($\text{FC}_{\log 2}$) and annotated the 10 most highly expressed DETs in non-healthy harbor porpoises

compared with that in healthy porpoises (Table 3). The most highly expressed transcript was TRIM63 ($FC_{\log 2} = 5.65$), a marker gene for muscle atrophy that, under amino acid starvation, induces proteasomal degradation of muscle protein (Rom & Reznick, 2016). Three transcripts were identified with their main function in metabolism (SLC2A1, $FC_{\log 2} = 4.83$; AMPD3, $FC_{\log 2} = 4.40$; CA7, $FC_{\log 2} = 4.68$). CA7 also has a role in oxidative stress and gluconeogenesis (Monti et al., 2017; Di Fiore et al., 2018). One transcript in endocytosis and monocyte adhesion (SORL1, $FC_{\log 2} = 4.58$), one transcript in ribosomal biogenesis (RRS1, $FC_{\log 2} = 4.50$), and two transcripts were involved in regeneration processes (CSRP3, $FC_{\log 2} = 4.43$; LYPD3, $FC_{\log 2} = 4.47$). Multiple transcripts (TNFRSF12A, $FC_{\log 2} = 4.37$; GADD45G, $FC_{\log 2} = 4.40$) were associated with response to various cell stressors.

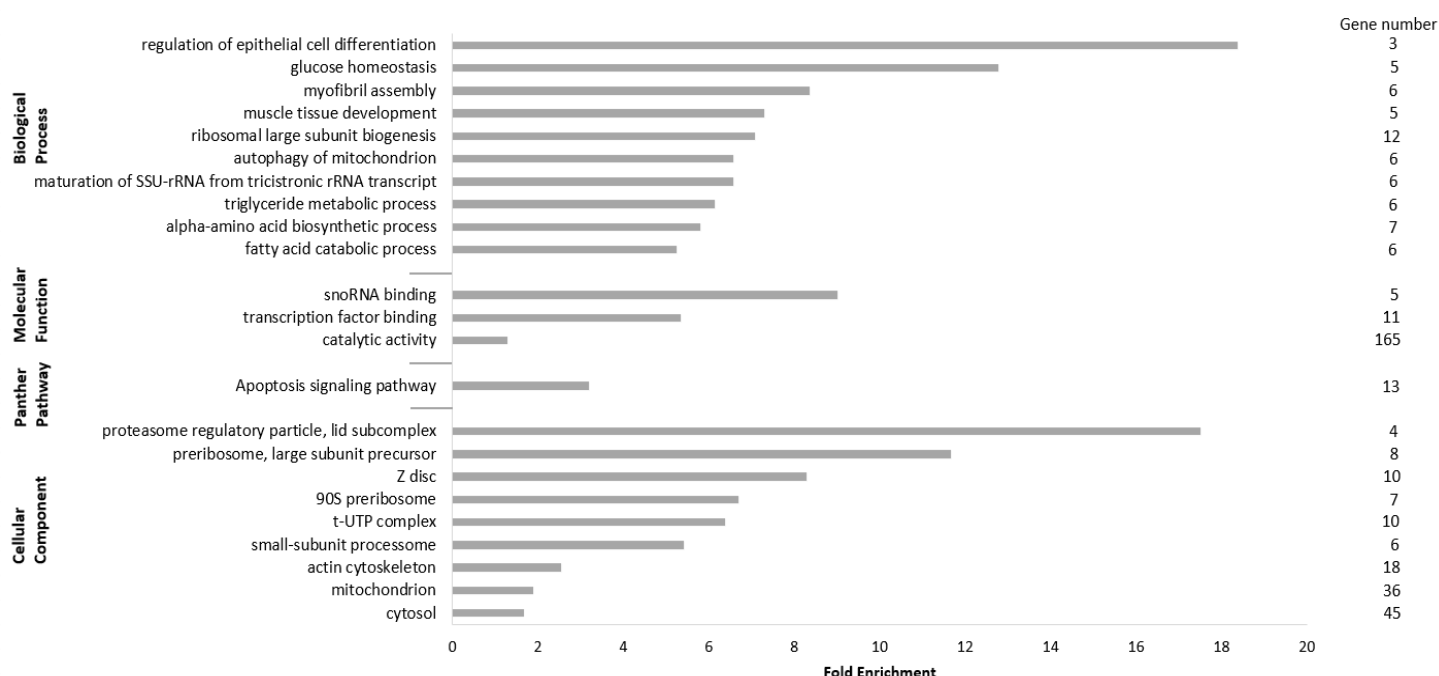


Figure 1 Panther analysis of the upregulated differentially expressed transcripts (DETs). Only DETs with the applied cutoffs fold change ($FC_{\log 2} \geq 1$, FDR-corrected p-value ($p\text{-value}_{FDR}$) ≤ 0.05 , and transcripts per million ($TPM_{\text{non-healthy}}$) ≥ 5 were considered for the analysis, resulting in 739 transcripts. Presented are the top 10 significantly upregulated terms of the GO Slim categories "Biological Processes", "Molecular Function", "Cellular Component", and "Pathways" with fold enrichment and number of genes.

Table 3 Top 10 of the differentially expressed transcripts with the highest fold change in the muscles of non-healthy harbor porpoises compared with that in the muscles of healthy harbor porpoises.

Gene ID	Transcript Name	FC _{log2}	p-value _{FDR}	TPM non-healthy	TPM healthy	Function
TRIM63	Tripartite Motif Containing 63	5.65	1.73×10^{-37}	2807.07	45.53	Regulates the proteasomal degradation of muscle proteins under amino acid starvation
SLC3A1	Solute Carrier Family 3 Member 1	4.83	9.10×10^{-21}	32.26	0.84	Membrane glycoprotein involved in neutral and basic amino acid transport
CA7	Carbonic Anhydrase 7	4.68	4.42×10^{-14}	27.09	0.64	Zinc metalloenzyme that catalyzes the reversible hydration of carbon dioxide
SORL1	Sortilin Related Receptor 1	4.58	6.46×10^{-13}	7.30	0.19	Plays role in endocytosis and sorting, promotes adhesion of monocytes
RRS1	Ribosome Biogenesis Regulator 1 Homolog	4.50	4.22×10^{-17}	60.91	2.08	Enables 5S rRNA binding activity and involved in mitotic metaphase plate congression
LYPD3	LY6/PLAUR Domain Containing 3	4.47	4.34×10^{-16}	109.69	3.91	Supports cell migration
CSRP3	Cysteine and glycine-rich protein 3	4.43	9.57×10^{-19}	977.57	38.66	Regulator of myogenesis
AMPD3	Adenosine Monophosphate Deaminase 3	4.40	3.17×10^{-18}	19.26	0.72	AMP deaminase with a role in energy metabolism
GADD45G	Growth Arrest And DNA Damage Inducible Gamma	4.40	2.80×10^{-19}	499.80	20.45	Involved in the regulation of growth and apoptosis and response to environmental stresses
TNFRSF12A	TNF Receptor Superfamily Member 12A	4.37	1.17×10^{-20}	361.56	14.67	Positive regulation of extrinsic apoptotic signaling pathway and regulation of wound healing, promotes angiogenesis

Logarithmic fold change (FC_{log2}), FDR-corrected p-value, and mean expression in transcripts per million (TPM) in non-healthy and healthy harbor porpoises and gene function are presented. For the description of functions, the database GeneCards version 5.14.0 was used

Downregulated transcripts in non-healthy harbor porpoise muscles are involved in cell adhesion and cell organization

For the analysis of enriched terms within the lowly expressed transcript set in muscles of non-healthy harbor porpoises compared with that of healthy porpoises, only DETs with FC_{log2} ≤ -1, p-value_{FDR} ≤ 0.05, and TPM_{non-healthy} ≥ 5 were considered (Table 4; see Supplementary data).

Here, we could identify development-related biological processes (anatomical structure development, $FE = -2.48$) and molecular functions associated with adhesion and binding (collagen binding, $FE = -40.85$; actin binding, $FE = -4.93$) significantly less enriched in muscles of non-healthy harbor porpoises compared with those of healthy porpoises (Figure 2).

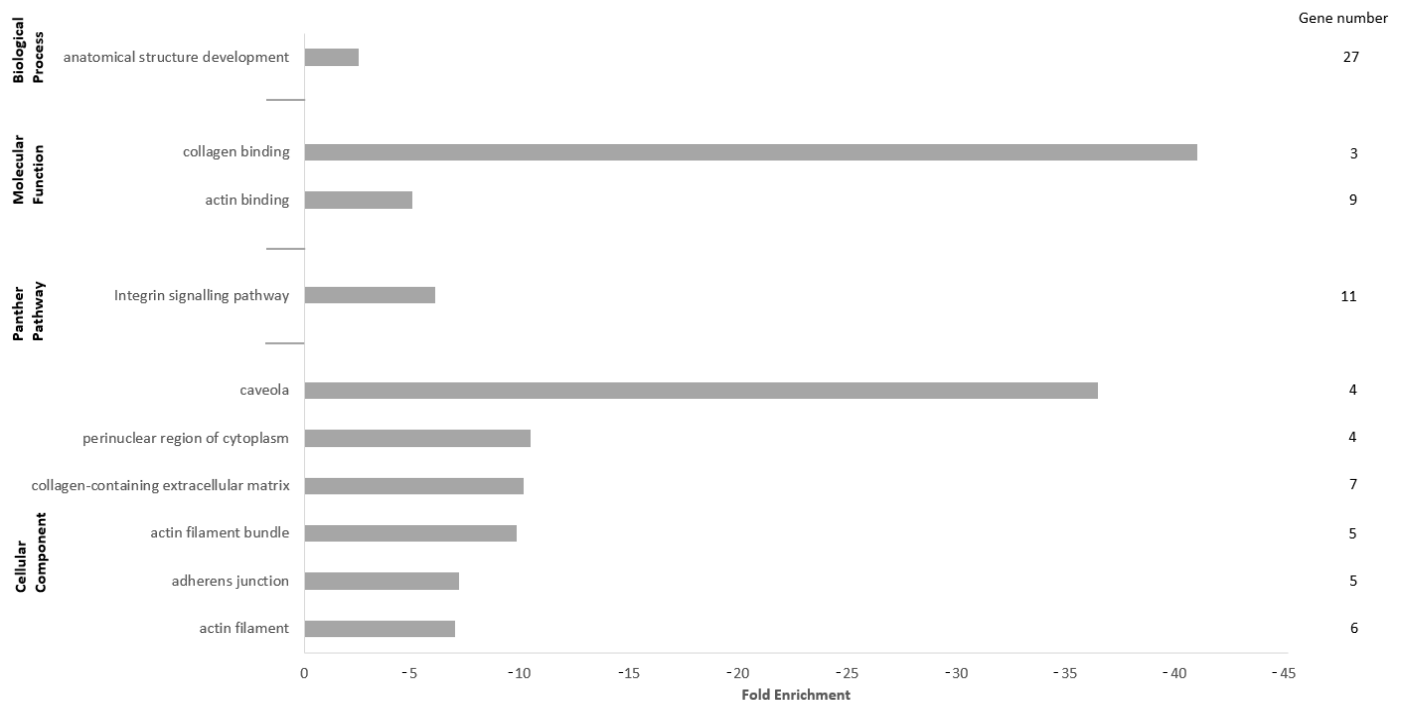


Figure 2 Panther analysis of the downregulated differentially expressed transcripts (DETs). Only DETs with the applied cutoffs fold change ($FC_{\log 2}$) ≤ -1 , FDR-corrected p-value ($p\text{-value}_{FDR}$) ≤ 0.05 , and transcripts per million ($TPM_{\text{non-healthy}}$) ≥ 5 were considered for the analysis, comprising 194 genes. Presented are the top 10 significantly upregulated terms of the GO Slim categories “Biological Processes”, “Molecular Function”, “Cellular Component”, and “Pathways” with fold enrichment and number of genes.

The top 10 downregulated DETs were also further examined (Table 4). Within the lowest expressed transcripts in muscles of non-healthy harbor porpoises compared with those of healthy animals, the least expressed transcript was UCP3 ($FC_{\log 2} = -4.60$). It is primarily expressed in skeletal muscle and protects from lipid-induced oxidative stress in mitochondria. One transcript involved in neural regeneration (NREP, $FC_{\log 2} = -3.94$) was found less expressed. Several transcripts that are associated with extracellular matrix organization and remodeling and membrane structure (COL1A2, $FC_{\log 2} = -3.88$; COL1A1, $FC_{\log 2} = -3.71$; TMOD4, $FC_{\log 2} = -2.89$) were among the top 10 downregulated DETs. CA3 ($FC_{\log 2} = -2.98$), an isoform of CA7, was identified and also catalyzes the hydration of carbon dioxide, although with a lower efficiency than CA7 (Monti et al., 2017). ANGPTL1 ($FC_{\log 2} = -2.74$) showed a low expression and is the only known vascular endothelium growth factor. One pro-apoptotic (G0S2, $FC_{\log 2} = -2.74$), one anti-proliferative transcript interacting with retinoid acid (RXRG, $FC_{\log 2} = -2.80$), and one involved in cartilage scaffolding and insulin signaling (CILP, $FC_{\log 2} = -2.58$) were identified among the lowest expressed transcripts.

Table 4 Top 10 of the differentially expressed transcripts with the lowest fold change in the muscles of non-healthy harbor porpoises compared with that in the muscles of healthy harbor porpoises.

Gene ID	Transcript Name	FC _{log2}	p-value _{FDR}	TPM non-healthy	TPM healthy	Function
UCP3	Uncoupling Protein 3	-4.60	3.54×10^{-18}	16.85	596	Primarily expressed in skeletal muscle, protects mitochondria against lipid-induced oxidative stress
NREP	Neuronal Regeneration Related Protein	-3.94	1.18×10^{-13}	8.95	190.54	Involved in neural regeneration
COL1A2	Collagen Type I Alpha 2 Chain	-3.88	5.81×10^{-26}	17.16	316.29	Fibril-forming collagen in most connective tissues
COL1A1	Collagen Type I Alpha 1 Chain	-3.71	1.60×10^{-21}	12.63	208.81	Fibril-forming collagen in most connective tissues
CA3	Carbonic Anhydrase 3	-2.98	4.15×10^{-12}	109.02	1032.67	Catalyze the reversible hydration of carbon dioxide
TMOD4	Tropomodulin 4	-2.89	2.65×10^{-9}	63.53	648.99	Actin filament organization, muscle contraction, and myofibril assembly
RXRG	Retinoid Receptor Gamma X	-2.79	4.73×10^{-7}	10.50	90.65	Mediates antiproliferative effects of retinoic acid
ANGPTL1	Angiopoietin Like 1	-2.74	1.97×10^{-8}	8.70	71.08	Growth factor largely specific for vascular endothelium
G0S2	G0/G1 Switch 2	-2.74	2.00×10^{-5}	42.09	374.66	Positive regulation of extrinsic apoptotic signaling pathway
CILP	Cartilage Intermediate Layer Protein	-2.58	1.66×10^{-9}	7.98	57.68	Cartilage scaffolding

Logarithmic fold change (FC_{log2}), FDR-corrected p-value, and mean expression in transcripts per million (TPM) in non-healthy and healthy harbor porpoises and gene function are presented. For the description of functions, the database GeneCards version 5.14.0 was used.

Moreover, important transcripts involved in gluconeogenesis and glycolytic metabolism were found differentially expressed in the filtered set of highly and less expressed transcripts (Figure 3; see Supplementary data). Upregulated transcripts played key roles in catalyzation of acetyl-coA (ACSS1, FC_{log2} = 1.10), production of glucose from lactate (PCK2, FC_{log2} = 2.39), and initiation of the first step of the glucose metabolism (HK2, FC_{log2} = 1.90). Downregulated transcripts were involved in regulation of gluconeogenesis (FBP2, FC_{log2} = -1.78), glycolytic production of ATP (PGK1, FC_{log2} = -1.40), breakdown and synthesis of glucose (PGM1, FC_{log2} = -1.64), or key steps of glycolysis (ALDOA, FC_{log2} = -1.52).

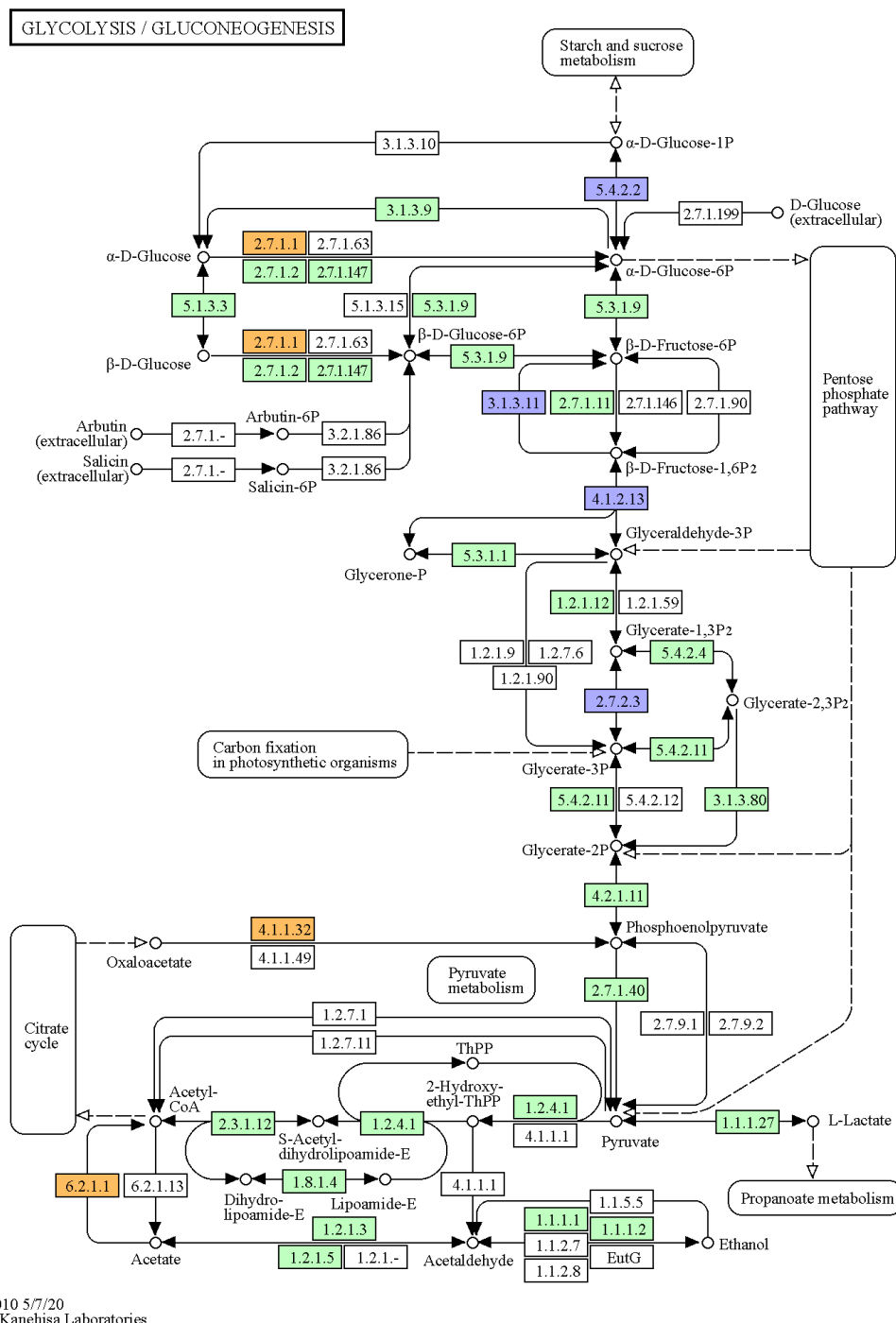


Figure 3 Glycolysis/gluconeogenesis Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of the human (hsa00010). Marked in orange are identified upregulated DETs in muscles of non-healthy harbor porpoises (2.7.1.1, HK2; 4.1.1.32, PCK2; 6.2.1.1, ACSS1), and marked in violet are here identified downregulated DETs (5.4.2.2, PGM1; 3.1.3.11, FBP2; 4.1.2.13, ALDOA; 2.7.2.3, PGK1). The green colored genes stand for identified genes of the pathway in the human.

Verification of transcriptome analysis in qRT-PCR of selected candidate genes

Because of the limited sample set of 2 per group for the transcriptome analysis, we verified the differential gene expression results by qRT-PCR in additional muscle samples of non-healthy and healthy harbor porpoises. A total of 18 candidate genes were chosen on the basis of

differences in expression between the two conditions (upregulation): abundance of transcripts and involvement in GO Slim “Biological Process” terms (Supplementary Table 3). Only upregulated and higher expressed transcripts were considered as candidate genes as they are likely involved in the active response to the possible lack of oxygen and resulting negative consequences. For the normalization of the qRT-PCR data, we tested three reference genes, namely, RPLP1, RPLP0, and EF2, of which RPLP1 was the most stable reference gene and selected for normalization. In addition, few of the candidate genes have been mentioned in other whale or seal studies, hinting at a possible aquatic adaptation in marine mammals but have not been examined in more detail (FOSL1, GADD45G, GLUL, HIPK2, IGFR1, NPY, PCK2, SIK1, TOLLIP, and TNFRSF12A). Of the chosen candidate genes, one could not be verified in qRT-PCR (TNFRSF12A). Because of a high content of Guanine/Cytosine (GC) and a high number of repetitive bases, it was not possible to generate an adequate primer pair. All candidate genes that were found upregulated in the RNA-Seq also had a higher relative FC (rel. FC) in non-healthy harbor porpoises in the qRT-PCRs (Figure 4; see Supplementary data). We could confirm upregulation of transcripts involved in oxidative stress response and antioxidant defense in muscles of non-healthy harbor porpoises (HIPK2, mean rel. $FC_{\text{non-healthy}} = 14.60$; CTH, mean rel. $FC_{\text{non-healthy}} = 10.05$; GADD45G, mean rel. $FC_{\text{non-healthy}} = 36.93$; MAFF, mean rel. $FC_{\text{non-healthy}} = 13.86$) of which two also possess antimicrobial properties (NPY, mean rel. $FC_{\text{non-healthy}} = 17.23$; TOLLIP, mean rel. $FC_{\text{non-healthy}} = 12.34$). Muscles of non-healthy harbor porpoises were confirmed to express higher degradation-associated genes (TRIM63, mean rel. $FC_{\text{non-healthy}} = 116.88$; SQSTM1, mean rel. $FC_{\text{non-healthy}} = 15.67$). Multiple candidate genes with a role in muscle regeneration and development were also found highly expressed in non-healthy harbor porpoises compared with that in healthy individuals (CSRP3, mean rel. $FC_{\text{non-healthy}} = 1.22$; ANKRD2, mean rel. $FC_{\text{non-healthy}} = 14.69$; SIK1, mean rel. $FC_{\text{non-healthy}} = 10.93$; FOSL1, mean rel. $FC_{\text{non-healthy}} = 60.64$). We lastly tested candidate genes with a role in anaerobic and lipid metabolism in a larger subset of samples.

Upregulation of transcripts involved in gluconeogenesis and glycolysis (CA7, mean rel. $FC_{\text{non-healthy}} = 48.58$; PCK2, mean rel. $FC_{\text{non-healthy}} = 7.36$; HK2, mean rel. $FC_{\text{non-healthy}} = 3.23$), amino acid metabolism (GLUL, mean rel. $FC_{\text{non-healthy}} = 23.37$), and fatty acid metabolic pathways (IGF1R, mean rel. $FC_{\text{non-healthy}} = 5.95$; CA7) could be confirmed in qRT-PCR. The gene expression varied strongly between the single individuals for most analyzed candidate genes (Figure 4). The highest interindividual variation was found for TRIM63 (rel. FC max = 815.48, rel. FC min = 0.56), FOSL1 (rel. FC max = 410.41, rel. FC min = 0.18), CA7 (rel. FC max = 171.86, rel. FC min = 0.18), and GADD45G (rel. FC max = 171.29, rel. FC min = 0.69), whereas

the gene expression of CSRP3 was the most homogeneous among the non-healthy harbor porpoises (rel. FC max = 3.60, rel. FC min = 0.04).

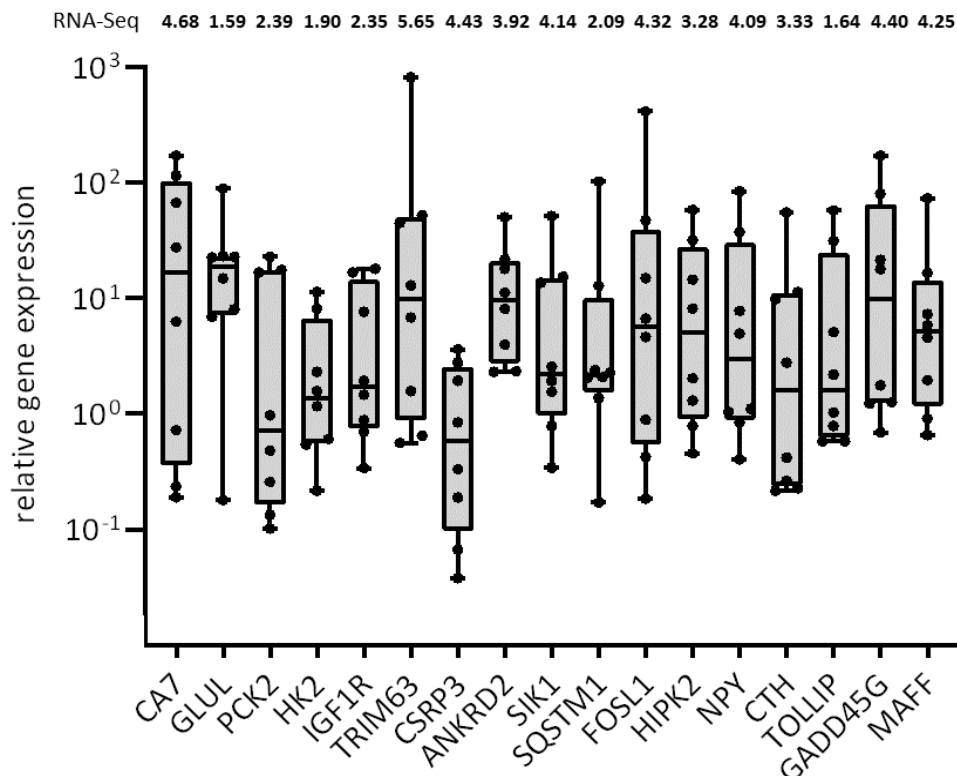


Figure 4 Boxplot with median of the relative gene expression of selected candidate genes in muscles of non-healthy harbor porpoises compared with that of healthy animals. The gene expression of the individual non-healthy harbor porpoises is represented by the dots ($n = 8$). The vertical y-axis is presented logarithmically due to the high inter-individual variation of the harbor porpoises. The fold changes (FC_{log2}) of the RNA-Seq for each transcript are indicated above.

Discussion

Harbor porpoises from the North and Baltic Seas show a higher level of parasitic infections and associated lesions in the respiratory tract compared with porpoises from Arctic waters (Siebert et al., 2006a, 2020; Wünschmann et al., 2001). This may lead to an impaired oxygen uptake and available oxygen for high energy- and oxygen-consuming organs such as the brain, heart, and muscles. The skeletal musculature is especially essential for the fully-aquatic living marine mammals. They not only enable locomotion and diving ability but also play an important role in oxygen and nutrient storage in cetaceans. To analyze whether and how muscles are affected, we generated transcriptomes of the muscles from two non-healthy harbor porpoises and compared them with the muscle transcriptomes of healthy porpoises. We performed GO analyses and identified significantly differentially regulated transcripts in non-healthy animals.

Last, we selected a set of 18 transcripts as candidate genes for verification of the results of the RNA-Seq in a larger subset of samples and for further analyses.

Enhanced transcripts involved in response to oxidative stress but not to hypoxia in muscles of non-healthy porpoises

The skeletal musculature is necessary for locomotion and consumes large amounts of energy and oxygen, which is taken up and distributed from the lungs. We hypothesized that the observed lung damages of the harbor porpoises reduce oxygen uptake and supply to the muscles and thus cause hypoxic conditions in the tissue. Contrarily to this, we could not find a defined response to hypoxia. We found transcripts (NPY) upregulated in non-healthy harbor porpoises that are assumed to enhance tissue perfusion (Mirman et al., 2020) and thus may help secure adequate oxygen distribution in the tissue and alleviate possible hypoxic states. In non-healthy harbor porpoises, we confirmed an elevated oxidative stress response (HIPK2, GADD45G, MAFF, CTH, and SLC3A1; Figures 1, 4; Table 3; Supplementary Table 3). Oxidative stress not only can be induced by free radicals such as ROS and xenobiotics but also form naturally during metabolic processes (Sies et al., 2022; Sies & Jones, 2020). Excessive concentrations of ROS can lead to apoptosis (Sies & Jones, 2020). HIPK2 is thought to act as a transcriptional switch, deciding between apoptosis or survival of the cell (de la Vega et al., 2012) and increases survival signaling when overexpressed (Li et al., 2018; Torrente et al., 2017). GADD45G, which can act as a stress sensor and is associated with oxidative stress, was also upregulated in muscles of non-healthy harbor porpoises, but has been found decreased in blubber of Northern elephant seals after prolonged stress conditions (Khudyakov et al., 2017; Turner et al., 2019).

The opposite regulation observed here may be due to different functions and, hence, regulation in the respective tissues. Three highly expressed transcripts in non-healthy harbor porpoises were associated with synthesis or regulation of the antioxidant glutathione (MAFF, CTH, and SLC3A1; Figure 1; Table 3; Supplementary Table 3). Whereas MAFF regulates glutathione concentrations (Wang et al., 2020), CTH and SLC3A1 are involved with the synthesis of glutathione by maintaining levels of its precursors cystine and cysteine (Cha et al., 2018; McBean, 2017; Wu et al., 2020). Glutathione is an important factor of the antioxidant defense by effectively clearing excessive reactive oxygen or nitrogen species and xenobiotics (Cantú-Medellín et al., 2011; García-Castañeda et al., 2017; Wilhelm Filho et al., 2002). Therefore, it may be more likely elevated due to high oxidative stress in the tissue and, additionally, exposure

to pollutants of the porpoises (Sies & Jones, 2020). Glutathione can exist in two states, namely, reduced (GSH) or oxidized (GSSG). The ratio between GSH to GSSG can be used as indicator of oxidative stress, with increased GSSG implying higher oxidative stress (Owen & Butterfield, 2010), which should be considered for future studies.

Transcriptome analysis hints at enhanced clearance and regeneration of muscle cells in non-healthy harbor porpoises

Oxidative stress and hypoxia are known to induce muscle atrophy by increasing proteolysis and inhibiting translation of proteins (Lian et al., 2022). However, multiple studies indicate that autophagy is indispensable for muscle recovery after injury, for timely clearance of cell debris and after intense exercise (Call & Nichenko, 2020; Xia et al., 2021). In muscles of non-healthy harbor porpoises, we found processes and transcripts upregulated that are associated with infections, degradation, and atrophic flux (Figure 1; Table 3). Several transcripts (NPY and TOLLIP; Figure 4) with antimicrobial properties were found (Anderson et al., 2022; Mancina et al., 2012). Although it is not possible to determine the specific cause, the upregulation may protect from an easily contractible, secondary bacterial infection due to an impaired immune system or act as a reaction to an already existing bacterial infection (Mancina et al., 2012; Siebert et al., 2001). Still, the muscle function of TOLLIP has not been fully described and remains partly hidden, highlighting it as a possible novel adaptation for further research (Boursereau et al., 2017). The qRT-PCR results (Figure 4) could confirm high expression in non-healthy harbor porpoises of muscle atrophy-associated transcript TRIM63 (Rom & Reznick, 2016; Thoma & Lightfoot, 2018) and of SQSTM1, which may be indicative of autophagic cargo flux (Puissant et al., 2012). Together, these result hint at a high degradation rate of debris, defective cells or misfolded proteins in non-healthy harbor porpoises. Interestingly, we found elevated ribosome biogenesis (RRS1, Table 3; “ribosomal large subunit biogenesis” and “maturation of SSU-rRNA from tricistronic rRNA transcript”, “snoRNA binding”, Figure 1), despite it being one of the first inhibited processes upon injury, as it is highly energy-demanding (Shah et al., 2013). Furthermore, we found enhanced regenerative processes and confirmed elevated expression of associated transcripts (CSRP3, ANKRD2, SORL1, FOSL1, and SIK1; Table 3; Figure 4; see Supplementary data). Two highly expressed transcripts were found to promote myogenesis (SIK1) and function as master regulator of muscle function and development (CSRP3; Mutryn et al., 2015; Stewart et al., 2013; Williams et al., 2021). CSRP3 can also interact with Ankyrin Repeat Domains (ANKRD2, Supplementary Table 3), which are transcriptional stress responders and upregulated under

oxidative stress (Belgrano et al., 2011; Cenni et al., 2019; Tsompanidis et al., 2016). FOSL1, an early response gene that mediates muscle injury, has also been found upregulated in the brain of hooded seals after reoxygenation (Hoff et al., 2017), so it may support perfusion of the muscle tissue and prevention of ROS. Although we could confirm elevated degradation processes in muscles of non-healthy harbor porpoises, we could also confirm unexpectedly enhanced regeneration and development, which may point to an adaptive mechanism in harbor porpoises. A precautionary high baseline expression of transcripts involved in muscle regeneration and development may ensure fast replacement of degraded cells, support tissue function, and prevent tissue loss observed in other mammals (Nixon et al., 2016; Xia et al., 2021; Yadava et al., 2016). However, protein turnover is a very costly process, and, therefore, its elevation may strongly affect the maintenance of the muscles and overall energy balance of the porpoises.

Dysregulation of lipid and glucose metabolism for high energy processes in muscles of non-healthy harbor porpoises

Stress such as hypoxia and ROS can affect metabolism pathways that have been continuously suggested for and investigated in marine mammals (Fair & Becker, 2000; Horscroft & Murray, 2014; Khudyakov et al., 2015a). Whales rely heavily on carbohydrate and lipid energy production, changing to aerobically metabolized lipids as preferred energy source under stress and reduced food uptake (Chicco et al., 2014; Kanatous et al., 2008; Sierra et al., 2015; Velten et al., 2013). In muscles of non-healthy harbor porpoises, we identified differently regulated lipid and anaerobic metabolic pathways like “glucose homeostasis”, “triglyceride metabolic process”, and “fatty acid catabolic process” (Figure 1; Table 3). Transcripts indicating a dysregulation of glucose and lipid metabolism were identified and confirmed in non-healthy porpoises (AMPD3 and SQSTM1, Table 3; Supplementary Table 3; Calvo-Garrido et al., 2019; Caspi et al., 2020; Hong et al., 2017). In concert with this, we found Insulin like growth factor (IGF) receptor IGF1R upregulated, which enhances survival and affects lipid homeostasis (Houser et al., 2013; Li et al., 2022; O’Neill et al., 2016). In addition, transcripts protecting from lipid-induced oxidative injury were found downregulated in non-healthy harbor porpoises (UPC3, Table 4), potentially hinting at a higher capacity of aerobic lipid metabolism (Burns et al., 2010; Polasek et al., 2006). Moreover, gluconeogenic transcripts were found downregulated, whereas mostly glycolytic transcripts were found upregulated (Figure 2). Interestingly, ALDOA, which can indicate glycolytic metabolism rates (Hoff et al., 2017), was found downregulated (Figure 3; Supplementary data). However, other metabolic rate-limiting

transcripts were confirmed to be highly expressed (Figures 3, 4) and are also involved in production of glucose from lactate (PCK2 and HK2, Figure 3; Champagne et al., 2012; Nedvedova et al., 2018), in glutamate/glutamine synthesis, which can be used for high energy processes (GLUL, see Supplementary data; de Theije et al., 2018; Rogeri et al., 2020) and with a function in lipogenesis and gluconeogenesis (CA7, Table 3; Supplementary data; Di Fiore et al., 2018; Monti et al., 2017). Although, CA7 is the most efficient catalytic carbonic anhydrases, it is still one of the least researched isoforms and has not been studied extensively in marine mammals (Chegwidden & Carter, 2000; Cincinelli et al., 2011; Tower & Young, 1973; Yang et al., 2000). A high reliance on glycolysis has also been observed in marine mammals under long-term stress (Champagne et al., 2012; Fabrizius et al., 2016; Park et al., 2015; Tian et al., 2017; Torres-Velarde et al., 2021), which may also be true in response to ROS stress and maintenance of high energy functions such as translation and regeneration of energy-consuming muscles observed in this study.

Conclusion

This study is the first describing the muscle transcriptomes of the harbor porpoise. Here, we investigated the muscle transcriptomes of two German harbor porpoises suffering from lesions in the respiratory tract and bronchopneumonia and compared them with muscle transcriptomes of healthy porpoises to analyze adaptations to reduced oxygen levels. We could find hints at adaptations like a possibly finely tuned switch between swift degradation of cell debris or misfolded proteins and tissue regeneration. Glutathione-associated transcripts have been found upregulated in muscles of the non-healthy harbor porpoises and may point to a high reliance on this antioxidative gene. Hints at a higher capacity for glycolysis and higher reliance on lipid metabolism have been found in muscles of non-healthy harbor porpoises. This may be utilized by the high-energy consuming muscle to ensure additional highly consuming processes like regeneration and translation. Our findings suggest that harbor porpoises do not suffer from hypoxic conditions in the muscles exacerbated by pathological lesions in the lung but may experience elevated oxidative stress caused by excessive ROS. Although the function of the identified transcripts has to be further analyzed in future studies, this study presents an important step to better understand the adaptations to stresses of cetaceans on a molecular level.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

Ethical review and approval were not required for the study since the investigated animals were collected once they were dead by stranding or by-caught within the German stranding network. The German stranding network conducts work such as collecting and holding carcasses and samples from European protected species following appropriate licenses from the relevant authorities.

Author contributions

ED, US, and AF conceived the research idea. US provided the samples and additional data. ED and AF derived the experimental procedure. ED performed the experiments and data analysis and wrote the manuscript with input from all authors. All the authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at:
<https://www.frontiersin.org/articles/10.3389/fmars.2023.1232305/full#supplementary-material>

6 Chapter II

Transcriptomic insights into the immune responses of the lung and muscle of non-healthy harbor porpoises (*Phocoena phocoena*)

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Abstract

Introduction: The harbor porpoise populations of the North and Baltic Seas are highly impacted by human activities, including underwater-radiated noise, fisheries and pollution. These cumulative stressors can have various detrimental effects, such as reduced foraging success, altered behavior and an impaired immune system. Harbor porpoises especially suffer from diseases of the respiratory tract which are partly caused or exacerbated by high parasitic prevalence in the lungs that may ultimately affect diving ability and competitiveness due to insufficient oxygen uptake and supply to the locomotor musculature.

Methods: To investigate pathophysiological mechanisms and potential compensatory adaptations to pathogenic insults, we employed transcriptomics and compared lungs and muscles of harbor porpoises with compromised respiratory health to healthy individuals. Additionally, a *de novo* transcriptome assembly was generated to identify transcripts that may be involved in immune-related responses.

Results and Discussion: Non-healthy harbor porpoises showed a distinct host-pathogen defense reaction in the lung, suggesting similarities to immune responses of humans suffering from lung diseases, which may be conserved along the mammalian lineage despite vastly different habitats. However, the lung transcriptomes did not indicate a Th2 immune response which is typically activated upon parasitic insults. Severely infected harbor porpoises may be overwhelmed or weakened by prolonged parasitic exposure and immune activation, possibly affecting simultaneous pathogenic clearance and tissue repair. The muscles of non-healthy harbor porpoises exhibited enhanced stress signaling and tightly regulated tissue degradation/regeneration, potentially reflecting a chronic inflammation state. Higher expression of hypoxia- and oxidative stress-associated transcripts in the muscle were consistent with hypoxia-induced transcriptional patterns and suggest a systemic pathological challenge. The *de novo* assembly identified significantly dysregulated non-coding RNAs in the lung and muscle which may be associated with regulatory processes. Several transcripts of the *de novo* assembly remained unidentified, thus their putative function needs to be elucidated. In marine mammals, the understanding of molecular immune responses still remains incomplete. This is the first study to describe the lung transcriptome of wild harbor porpoises in regard to pathophysiology. These insights contribute to the understanding of the interaction between anthropogenic impacts, infectious diseases and

molecular immune responses in cetaceans, thus supporting cetacean health assessments and conservation efforts.

KEYWORDS *de novo* assembly, harbor porpoise *Phocoena phocoena*, immune system - respiratory tract, lung, marine mammals, muscle, pathophysiology, transcriptome

Introduction

The harbor porpoise (*Phocoena phocoena*) is one of the most common and widely distributed cetacean species in European seas, and the only one native to German waters. While not of concern on a global stage, subpopulations, such as the Baltic Sea Proper harbor porpoises, are indexed on the IUCN Red List of Threatened Species (Amundin et al., 2022; Carlström et al., 2023). Recent studies have also noted a worrying decline in porpoise numbers for the German part of the North Sea (Nachtsheim et al., 2021). Due to their preference for coastal habitats, harbor porpoises are constantly exposed to natural and human-made environmental changes (Carlén et al., 2021). The North and Baltic Seas are notoriously frequented by maritime traffic and experience a surge in recreational and industrial interest (e.g., offshore construction, military and seismic surveys; Erbe et al., 2019; Kastelein et al., 2018; Schaffeld et al., 2022; Siebert et al., 2013, 2022). Chemical and industrial flow-off can wash contaminants and toxic metals into the sea which biomagnify in apex predators such as the harbor porpoise (Jepson et al., 2005; Siebert et al., 1999; Sonne et al., 2020b). Bioaccumulation of these reagents has been suspected to impair the reproduction of harbor porpoises (Murphy et al., 2015; Wells et al., 2005; Williams et al., 2021). Additionally, neonates are impacted to some degree, as females offload a considerable amount of accumulated contaminants to their calves (van den Heuvel-Greve et al., 2021). The continuous exposure to contaminants also impairs the immune system by acting as immunosuppressant and hence, promotes pathogen contraction and inhibits immune defense responses (Desforges et al., 2016; Siebert et al., 2009; Sonne et al., 2020b). In the last decades, harbor porpoises from German waters have shown declining health states compared to Arctic and Scandinavian populations that live in less humanly frequented areas (Lehnert et al., 2014; Siebert et al., 2001, 2009, 2020). While harbor porpoises may be able to cope with mild levels of parasitic infestation, they show increasingly broader parasitic diversity and load (Ryeng et al., 2022; Sonne et al., 2020a). Especially the respiratory tract seems to be

targeted which exhibits accumulating pathological and inflammatory lesions that are associated with lungworm prevalence and contraction of secondary bacterial infections (IJsseldijk et al., 2021; Jepson et al., 2000; Siebert et al., 2006a, 2009, 2020; Wünschmann et al., 2001). Studies have hypothesized that such parasitic infestations and pathological lesions may hinder lung function due to tissue damage and parasitic obstruction of air and blood flow (Ceco et al., 2017; Siebert et al., 2001, 2020; Ten Doeschate et al., 2017).

With harbor porpoises only utilizing approximately 60% of their complete lung capacity (Rojano-Donate et al., 2018, personal communication with U. Siebert), further impairment of the lung tissue function may severely aggravate their ability to compensate these detrimental impacts. Although harbor porpoises tend to preferably dive shallow and short (Rojano-Donate et al., 2024), telemetry studies have observed dives up to 200–400 m, if the bathymetry allows for such depths (Nielsen et al., 2018; Westgate et al., 1995). These deeper dives are usually performed to forage (Rojano-Donate et al., 2024; Wisniewska et al., 2018), or spontaneously to avoid excessive underwater-radiated noise (Elmegaard et al., 2023; Frankish et al., 2023). Deterrence by exceeding noise levels can also cause a prolonged stay at deeper sea levels (Elmegaard et al., 2023; Wisniewska et al., 2018), which may put stressed harbor porpoises at higher risk of gas embolism (reviewed in Fahlman, 2024), if oxygen levels are insufficient to support unplanned, breath-hold dives (Williams et al., 2020).

Studies in humans have confirmed negative effects on the muscle stemming from diseases of the lung, such as bronchopneumonia, chronic obstructive pulmonary disease (COPD) and pulmonary fibrosis (Ceco et al., 2017; Gea et al., 2013; Jaitovich & Barreiro, 2018). While major immune responses often are conserved among the mammalian lineage, marine mammals may possess differing mechanisms to adapt to their life in the aquatic environment (Di Guardo et al., 2019; Haley, 2003).

In order to understand the described mechanism better, we performed a comparative transcriptome analysis of the lung and main locomotor muscle of wild harbor porpoises from the North and Baltic Seas against the recently published harbor porpoise reference genome (Scottish population; Davison et al., 2025). We compared harbor porpoises with impaired and good respiratory health to further understand molecular compensatory mechanisms for reduced oxygen uptake caused by respiratory lesions. Additionally, we generated a multi-tissue *de novo* transcriptome assembly (brain, lung, liver and muscle) to detect novel transcripts that were potentially involved adaptive immune and stress responses.

Materials and methods

Animals and sampling

The harbor porpoises used in this study stranded dead or died after live-stranding or as bycatch between 2016 and 2022 in Schleswig-Holstein, Germany. Tissue samples were collected during routinely conducted necropsies at the Institute of Terrestrial and Aquatic Wildlife Research (ITAW), University of Veterinary Medicine Hannover, Foundation, Büsum, Germany, which is part of the German stranding network (Benke et al., 1998; Siebert et al., 2006b). Full necropsies and further investigations were conducted on all individuals according to standardized protocols (IJsseldijk et al., 2019; Siebert et al., 2001). Based on the summary of findings, animals were categorized into healthy (n = 5) and non-healthy (n = 13) individuals with regard to their pulmonary health status. Non-healthy animals displayed pathological lesions due to moderate or severe lungworm infestations and bacterial infections and suffered or died from bronchopneumonia (Table 1). Healthy individuals were in overall good pulmonary health (Table 1). Tissue samples of the skeletal muscle (*Musculus longissimus dorsalis*), lung, liver and brain (cerebellum and visual cortex) were either subsampled from archived samples or freshly sampled and immediately preserved in RNA stabilization solution (NucleoProtect RNA, Macherey-Nagel, Düren, Germany). All samples were then stored at -80 °C until subsequent usage.

RNA isolation and quality control

Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. The initial homogenization step with the kit's RLT buffer was replaced with a phenol-chloroform extraction to maximize yield and purity of the isolated RNA. Tissue samples (20–30 mg) were minced and homogenized by bead beating in 1 mL of Trifast reagent (PEQLAB, Erlangen, Germany). RNA was isolated from the homogenates by phase extraction using chloroform and ethanol (70%). Thereafter, the kit manual was followed and an additional 15 minute on-column DNA I digest (Qiagen, Hilden, Germany) step was conducted as recommended. RNA concentration and quality, indicated by the RNA Integrity Number (RIN), was assessed using the Agilent TapeStation System (Agilent Technology, Santa Clara, CA, United States). RIN scores of the samples varied from 4.8 to 7.0.

Table 1 Metadata of the wild harbor porpoises used in this study.

Individual	Experiments	Condition	Sex	Sampling Year	Location	Age Class	By-catch	Nutrition	Disease
Pph_h1	qRT-PCR	healthy	male	2020	BS	neonate	no	u	Perinatal death
Pph_h2	RNA-Seq (muscle)	healthy	male	2020	BS	neonate	suspected	u	None
Pph_h3	RNA-Seq (lung, muscle), qRT-PCR	healthy	male	2021	BS	juvenile	yes	G	Mild inflammation in the lung, stomach and liver
Pph_h4	DNA, RNA-Seq (lung, muscle, brain), qRT-PCR	healthy	female	2022	NS	neonate	no	G	Perinatal death
Pph_h5	qRT-PCR	healthy	female	2022	BS	adult	no	G	Suspected septicemia (<i>P. multocida</i>)
Pph_nh1	RNA-Seq (muscle)	non-healthy	female	2019	BS	adult	suspected	M	Bronchopneumonia, hepatitis, adrenitis, final septicemia (<i>P. multocida</i>)
Pph_nh2	RNA-Seq (muscle), qRT-PCR	non-healthy	male	2019	NS	juvenile	no	M	Bronchopneumonia, gastroenteritis
Pph_nh3	DNA, RNA-Seq (lung, liver), qRT-PCR	non-healthy	male	2022	BS	juvenile	yes	G	Bronchopneumonia, gastritis
Pph_nh4	DNA, RNA-Seq (lung, muscle), qRT-PCR	non-healthy	female	2022	NS	adult	no	P	Meningoencephalitis, bronchitis, gastritis
Pph_nh5	RNA-Seq (muscle)	non-healthy	male	2022	BS	juvenile	yes	G	Bronchopneumonia, gastritis, endoparasitosis
Pph_nh6	RNA-Seq (muscle)	non-healthy	male	2021	BS	juvenile	yes	G	Bronchopneumonia, gastritis
Pph_nh7	RNA-Seq (muscle)	non-healthy	male	2020	NS	juvenile	no	P	Bronchopneumonia, dermatitis
Pph_nh8	DNA, RNA-Seq (muscle), qRT-PCR	non-healthy	male	2019	NS	adult	no	P	Bronchopneumonia, gastritis
Pph_nh9	qRT-PCR	non-healthy	female	2016	BS	adult	no	P	Septicemia (red murrain)
Pph_nh10	qRT-PCR	non-healthy	female	2016	BS	adult	yes	P	Bronchopneumonia

Pph_ nh11	qRT-PCR	non- healthy	male	2016	NS	juvenile	no	P	Cachexia, bronchopneumo nia
Pph_ nh12	qRT-PCR	non- healthy	female	2021	BS	adult	yes	G	Bronchopneu- monia, endoparasitosis
Pph_ nh13*	qRT-PCR	non- healthy	female	2022	BS		suspected		Bronchopneu- monia, hepatitis, nephritis, dermatitis

*Condition, sex, cause of illness and death was unknown during performance of experiments. Shown are the year of the necropsy (sampling year) and location, health condition after assessment including diagnosed diseases, sex, age class, nutrition state and bycatch. Further data includes the experiments for which the samples were used. DNA, De Novo Assembly; RNA-Seq, RNA-Sequencing; qRT-PCR, quantitative real-time PCR; BS, Baltic Sea; NS, North Sea; nd, not determined; P, poor; M, moderate; G, good.

Sequencing and quality trimming

The RNA-Seq library preparation (NEBNext Ultra II Directional RNA library prep kit for Illumina, New England Biolabs, Ipswich, MA, USA) for paired-end sequencing of 2 x 150 nt was generated from 5000 ng RNA. Sequencing was performed on an Illumina NextSeq 2000 platform (StarSEQ, Mainz, Germany) with an output of 25 million reads per sample. Initial sequence quality control and trimming were carried out on the Galaxy server (version 21.09) of the University of Hamburg Biology department. Quality control was assessed using the FastQC v 0.73 and MultiQC v 1.11 tool on Galaxy. The first 20 5'-terminal nucleotides and Illumina adapter sequences (mismatch count = 2, internal match = 10) were cropped from the raw reads with Trimmomatic v 0.38.1. Reads below the length of 20 nucleotides were discarded and a minimum average quality value of 20 was required for consideration.

The raw sequence files are available at the NCBI Sequence Read Archive (SRA) from (SRA BioProject ID: PRJNA977857).

Differential expression analysis via RNA-Seq

The trimmed sequences of the lung and muscle transcriptomes were mapped and aligned against the harbor porpoise reference genome (mPhoPho1.1, released June 2024, RefSeq Accession: GCF_963924675.1) using HISAT v 2.2.1 and featurecounts v 1.6.4 (for detailed metrics, see Supplementary Table 1). Individual transcripts per million (TPM) for each transcript were calculated with the Galaxy tool “Generate CPM, TPM, RPK” v 0.4.0 and mean values for the non-healthy and healthy animals were calculated. Differentially expressed transcripts (DETs) were determined using DESeq2 v 2.11.40.7 (Love et al., 2014).

Validation of the RNA-Seq via quantitative real time PCR

Due to the low sample size, results of the lung transcriptome analysis were validated via qRT-PCR for a panel of transcripts in a larger sample subset (healthy, $n = 4$ and non-healthy, $n = 9$; Table 1). Of these transcripts, three were significantly upregulated (JCHAIN, GPNMB, OLR1) and one downregulated (QPCT) in non-healthy harbor porpoises in the transcriptome analysis. Based on the transcriptome analysis, the ribosomal protein RPS8 was selected as reference transcript with no regulation for normalization. cDNA was synthesized from 1,000 ng of total RNA, using the RevertAid H-First Strand cDNA Synthesis Kit (Thermo Fisher, USA) and diluted with Ribonuclease-free water (1:1). qRT-PCR was performed on the ABI 7500 real-time PCR system with the Power SYBR Green master mix (Applied Biosystems, Germany) and the following species-specific primer sequences: JCHAINfor-5'-GATGAAGATGAAAGG ACTGTTC-3', JCHAINrev-5'- TCAGAGGAACAATAATT CTGATGT-3'; OLR1for-5'- GATAATCCAATTATCCCAGGTG-3', OLR1rev-5'- TCTGGTGATGAAGTTCCATTAG-3'; GPNMBfor-5'-CATGATGTGCTGAGCAATGAG-3', GPNMBrev-5'- GTGCAT CACTGGTCAGAAGT-3'; QPCTfor-5'- TCGTTGAAGA ATATTTTCAGACTCG-3', QPCTrev-5'- GATGTGCAGTTGA TGCCATCT-3'; RPS8for-5'- GCAAGACAAGGATCATTGATG-3', RPS8rev-5'- CTCAGGAGTCAGCTTGGC-3'. The qRT-PCR settings were adjusted to 95 °C for 15 sec, 58 °C for 60 sec, 72 °C for 30 sec and repeated for 40 cycles. Each sample was applied with three technical replications and relative fold change was calculated based on the $\Delta\Delta CT$ method (Livak & Schmittgen, 2001). Statistical analysis was performed in GraphPad Prism v 10. Mean $\Delta\Delta CT$ values were tested for normal distribution, and the Mann-Whitney-U test was conducted for *post-hoc* analysis and statistical significance testing.

Gene ontology analysis

For Gene Ontology (GO) and pathway analysis, only differentially expressed transcripts (DET) with an FDR-corrected p-value ($p_{\text{valFDR}} \leq 0.05$), a logarithmic fold change ($FC_{\log 2} \geq 1$ or ≤ -1) and a mean $TPM_{\text{non-healthy}} \geq 5$ were considered for the analysis. Significantly dysregulated transcripts were analyzed for overrepresentation with the PANTHER classification database (v 19.0, reference: human *Homo sapiens*). GO Slim “Biological Processes” were analyzed and child terms with the highest specification were regarded. Results were visualized with the R package of “ggplot2”. Kyoto Encyclopedia of Genes and Genomes (KEGG, v 114.0) pathway

analysis was conducted to visualize the differential regulation of transcripts in selected pathways.

***De novo* transcriptome assembly and annotation**

Trimmed transcriptomes of the lung, muscle, liver and brain with the best RIN score ($n = 1$ per tissue, Table 1) were chosen to generate the *de novo* transcriptome assembly, for which Trinity v 2.15.2 with the associated tools was used on the High-Performance Computing Linux-Cluster of the University of Hamburg. Pre-assembly quality was checked with FastQC v 0.73 and MultiQC v 1.11 and post-assembly quality was analyzed with BUSCO v 5.6.1 against the databases of Mammalia and Cetartiodactyla. The annotation of the *de novo* assembly was generated with Trinotate v 4.0.2. Blast v 2.16.0 and Diamond v 2.1.11 were used to identify nucleotide- and protein sequence hits against multiple databases (Human EMBL Protein database, UniProt Protein database, UniProt Protein database for the bottlenose dolphin *Tursiops truncatus*, beluga *Delphinapterus leucas*, vaquita *Phocoena sinus*, sperm whale *Physeter macrocephalus*, narwhal *Monodon monocerus*, Indo-Pacific humpback dolphin *Sousa chinensis* and Yangtze finless porpoise *Neophocaena asiaeorientalis*). Also, a cetacean-specific database was generated, for which all cetacean entries of the UniProt Proteome database were included. The transcriptomes of the lungs and muscles were back-mapped with BowTie2 v 2.3.0 against the *de novo* assembly. BowTie2 included RSEM as estimation method and was set to trinity mode with a paired-end reverse strand library. Trinity transcripts were analyzed for differential expression with the R package of DeSeq2 v 1.48.1 and then filtered with the following cut-offs: $p_{\text{valFDR}} \leq 0.05$, $FC_{\log 2} \geq 1$ or ≤ -1 and $\text{mean TPM}_{\text{non-healthy}} \geq 5$.

Significantly dysregulated Trinity transcript sequences with no annotation via the Trinotate pipeline were extracted and run against the core nucleotide database (core_nt) of NCBI nucleotide blast (Blastn, BLAST+ version 2.17.0) with the organism set to mammalia (taxid ID: 40674) and megablast (highly similar sequences) for optimization. Blast hits were considered reliable, if the query covered $\geq 50\%$ and the sequences shared $\geq 70\%$ identity.

Results

Enhanced immune defense and reduced ECM processes in the lung

With the harbor porpoise genome as reference, over 17,591 transcripts were expressed in at least one individual. After the cut-offs were applied ($p_{\text{valFDR}} \leq 0.05$, $FC_{\log 2} \geq 1$ or ≤ -1 , mean $TPM_{\text{non-healthy}} \geq 5$), 66 upregulated and 10 downregulated DETs in the lungs of non-healthy harbor porpoises were considered for differential and pathway analyses (Figure 1A). Within the significantly dysregulated transcripts, the two non-healthy harbor porpoises exhibited varying expression profiles for a number of DETs (Figure 1B). In individual Pph_nh3, 17 DETs were higher expressed compared to individual Pph_nh4. These DETs were mostly involved in immune reactions, specifically immunoglobulin-mediated responses (Figure 1B, Supplementary Table 2). A total of 19 DETs showed higher elevation in individual Pph_nh4 and encompassed transcripts with a role in stress responses, metabolic processes and surfactant production (Figure 1B, Supplementary Table 2). Differentially expressed transcripts in the lungs of non-healthy harbor porpoises mostly showed significant elevation in immune system responses compared to healthy harbor porpoises (Figure 1C). Differentially expressed transcripts in the lungs of non-healthy harbor porpoises mostly showed significant elevation in immune system responses compared to healthy harbor porpoises (Figure 1C). This included leukocyte recruitment, immunoglobulin activation and responses to cytokine and inflammation (e.g., IGHG1, JCHAIN, CXCL8, IGKC, CXCL17, PIGR, Table 2; Figure 1C). Moreover, carbohydrate metabolism was enhanced in non-healthy harbor porpoises (Figure 1C, Table 2; AQP3, AQP7, CA4, SLC2A3; Supplementary Table 2). Several DETs were involved in metal ion binding, especially of iron (HP, IDO1, LTF, MOXD1, S100A12, S100A8, S100A9, STC2; Supplementary Table 2). KEGG pathway analyses of dysregulated transcripts in the lungs of non-healthy harbor porpoises showed high upregulation of transcripts involved in the proinflammation of lipid and atherosclerosis, a cardiovascular disease lipid and atherosclerosis, a cardiovascular disease (OLR1, NCF1, CXCL8, SELE, SELP, IL1B, CCL5, MMP3, MMP9; hsa05417, Supplementary Figure 1).

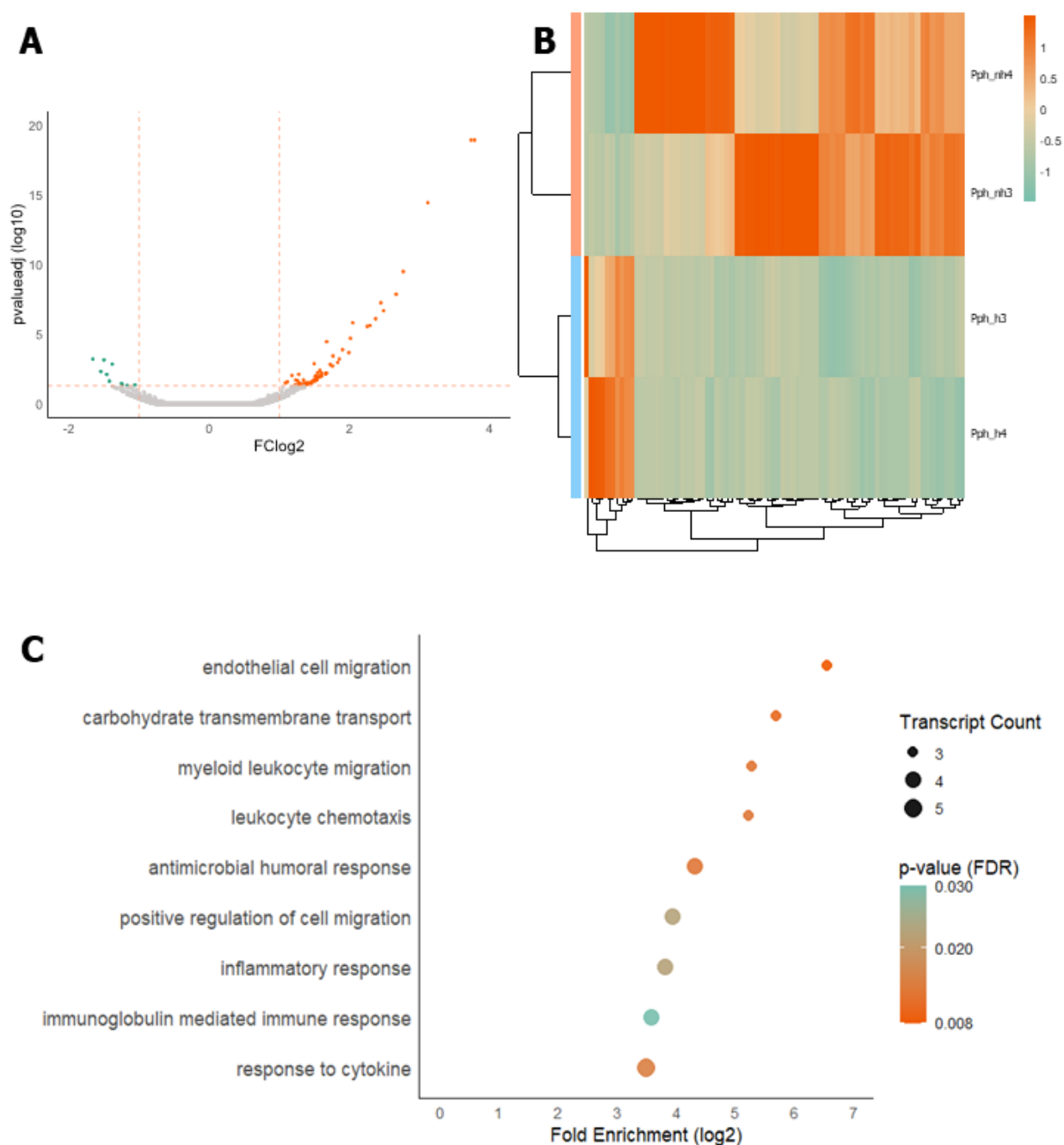


Figure 1 Differential analysis of the lung. **A** depicts the significantly dysregulated transcripts in the lung of non-healthy compared to healthy harbor porpoises. Upregulated transcripts are orange, downregulated transcripts are green. The x-axis represents the logarithmic fold change (FC_{log2}) and the y-axis indicates the FDR-corrected p-value. For better visualization, the y-axis has been log_{10} -transformed. Dashed orange lines highlight the applied cut-offs for fold change ($FC_{log2} \geq 1/\leq -1$) and for FDR-corrected p-value ($pvalue_{FDR} \leq 0.05$). Transcripts were considered significantly dysregulated when $pvalue_{FDR} \leq 0.05$, $FC_{log2} \geq 1/\leq -1$ and mean $TPM_{non-healthy} \geq 5$. **B** Hierarchical clustering of the significant DETs in non-healthy (apricot) and healthy (light blue) harbor porpoises. Upregulated transcripts are highlighted in orange and downregulated transcripts in green (see legend for FC_{log2} values). **C** Significant DETs were analyzed for overrepresented GO-Slim terms in the category “Biological Process” with PANTHER (reference: human). Significant enrichment could only be found for upregulated transcripts and are depicted here. The fold enrichment is log_2 -transformed and the transcript number of the identified terms are indicated by size of the dot. The color of the dots illustrates the significance level from high (orange) to low (green).

Table 2 Top 20 of the significant DETs in the lungs of non-healthy harbor porpoises.

Gene ID	Gene name	FC _{log2}	pval _{FDR}	Function (GeneCards/UniProt)
upregulated				
IGHG1 (LOC136143280)	Immunoglobulin Heavy Constant Gamma 1	3.79	1.16×10^{-19}	immune response
JCHAIN	Joining Chain Of Multimeric IgA And IgM	3.74	1.16×10^{-19}	immune response
CXCL8	Interleukin 8, C-X-C Motif Chemokine Ligand 8	3.12	3.64×10^{-15}	major mediator of the inflammatory response
IGKC (LOC136133810)	Immunoglobulin Kappa Constant	2.77	3.15×10^{-10}	immune response
IGHV3-23 (LOC136143286)	Immunoglobulin Heavy Variable 3-23	2.67	1.35×10^{-08}	immune response
LTF	Lactotransferrin	2.49	2.04×10^{-07}	major iron-binding and multifunctional, exocrine fluid protein
SFTPA (LOC136136182)	Surfactant Protein A	2.45	5.45×10^{-08}	essential for normal respiration by lowering surface tension
IGHV4-38-2 (LOC136143293)	Immunoglobulin Heavy Variable 4-38-2	2.38	7.72×10^{-07}	immune response
SPP1	Secreted Phosphoprotein 1, Osteopontin	2.30	2.37×10^{-06}	major non-collagenous bone protein
SFTPB	Surfactant Protein B	2.25	2.75×10^{-06}	essential for lung function and stability
MMP9	Matrix Metalloproteinase 9	2.05	1.48×10^{-06}	breakdown of ECM and collagens
TNC	Tenascin C	2.02	1.96×10^{-05}	extracellular matrix protein
TNFRSF17	TNF Receptor Superfamily Member 17	1.99	2.06×10^{-04}	humoral immune system, activation of NF-kappa-B and JNK
CCN5	Cellular Communication Network Factor 5	1.90	1.28×10^{-04}	bone turnover modulation
CXCL17	C-X-C Motif Chemokine Ligand 17	1.86	6.01×10^{-04}	mucosal, potent antimicrobial chemokine activity
PIGR	Polymeric Immunoglobulin Receptor	1.83	0.001	may be microbial scavenger
GPMB	Glycoprotein Nmb	1.77	3.66×10^{-04}	transmembrane glycoprotein (type I)
SLC2A3 (LOC136130342)	Solute Carrier Family 2 Member 3	1.73	0.001	glucose transporter
SELP	Selectin P	1.68	3.41×10^{-05}	immune responsive cell adhesion molecule
SBSN	Suprabasin	1.68	0.006	located in extracellular exosome
downregulated				
NREP	Neuronal Regeneration Related Protein	-1.66	0.001	may be involved in neural function and regulate alveolar generation
B2M (LOC136118455)	Beta-2-Microglobulin	-1.54	0.005	antigen presentation
NRXN1	Neurexin 1	-1.50	0.001	cell-cell-interaction, signal transmission
ADAMTS2	ADAM Metalloproteinase	-1.46	0.008	processes procollagens

With Thrombospondin Type 1 Motif 2				
KCP	Kielin Cysteine Rich BMP Regulator	-1.42	0.023	may be involved in positive regulation of BMP signaling pathway
SPON1	Spondin 1	-1.38	0.001	major factor for vascular smooth muscle cell, cell adhesion
MFAP2	Microfibril Associated Protein 2	-1.25	0.034	major antigen of elastin-associated microfibrils
COL3A1	Collagen Type III Alpha 1 Chain	-1.24	0.043	fibrillar collagen
BCHE	Butyrylcholinesterase	-1.17	0.048	detoxification of chemicals
TF (LOC136122826)	Transferrin	-1.06	0.043	iron transport

Given are the associated gene ID and gene name, logarithmic fold change ($FC_{\log 2}$), the FDR-corrected p-value ($pval_{FDR}$) and short description of the main function. If the gene IDs for the transcript were identified as a locus (LOC) in the transcriptomic analysis, they are given in the brackets. The associated gene ID was inferred via the databases of NCBI Gene and UniProt. For the short description of the main function, the databases of UniProt and GeneCards were used. Only 10 DETs were identified as significantly downregulated with the cut-offs $pval_{FDR} \leq 0.05$, $FC_{\log 2} \leq -1$ and mean $TPM_{non-healthy} \geq 5$.

Also, among the most significantly upregulated transcripts, multiple S100 transcripts (S100A4, S100A8, S100A9, S100A12), surfactant transcripts (SFTPA, SFTPB) and extracellular matrix-associated transcripts (MMP9, TNC, CCN5, SPP1, Table 2; MMP3, TIMP1, THBS4, Supplementary Table 2) were significantly higher expressed in the lungs of non-healthy harbor porpoises. No overrepresented biological processes could be identified for the significantly downregulated DETs in the lung, as the number of DETs was too low (Table 2). Five downregulated DETs were involved in extracellular matrix processes (ADAMTS2, KCP, SPON1, MFAP2, COL3A1; Table 2).

qRT-PCR of dysregulated transcripts supports the lung transcriptome results

We performed a qRT-PCR on selected transcripts to confirm the transcriptome results in a larger sample size. Mean relative gene expression of JCHAIN, GPNMB and OLR1 was higher in non-healthy compared to the healthy harbor porpoises (Supplementary Figure 2). QPCT showed lower expression in non-healthy harbor porpoises (Supplementary Figure 2). As expected with wild, unmanaged animals, the relative gene expression of all transcripts showed some individual variation within the condition groups. However, the relative gene expression showed a similar regulation in non-healthy and healthy harbor porpoises as the gene expression calculated in the transcriptome analysis.

Increased stress responses and reduced tissue structure maintenance in the skeletal muscles

In the skeletal muscle, 17,540 transcripts were expressed in at least one animal. Of these, a total of 525 DETs were significantly higher expressed in the skeletal muscle of non-healthy harbor porpoises, while 115 DETs showed a reduced expression compared to healthy individuals (Figure 2A). One bycaught, juvenile harbor porpoise, categorized as non-healthy (Pph_nh6), clustered with the healthy cohort, when comparing overall individual expression profiles of the significant DETs (Figure 2B).

Most enhanced pathways were associated with selective degradation processes (autophagy of mitochondrion, autophagosome assembly, protein ubiquitination, ubiquitin-dependent protein catabolic process; Figure 2C). Few regenerative processes, such as cardiac muscle cell differentiation, ribosomal large subunit biogenesis and rRNA processing, were elevated (Figure 2C). Also, muscles of non-healthy harbor porpoises displayed upregulation of processes pointing to nutrient stress, including glucose homeostasis and response to starvation. Likewise, several highly upregulated transcripts had a function in metabolism (AMPD3, IP6K3, PRODH, IRS2, PFKFB3, Table 3), with many involved in lipid metabolism (e.g., ADIPOR2, FASN, LPIN1, ACSL4, GPAT4, Supplementary Table 3). Additionally, a considerable number of DETs played a role in the nutrient stress-mediated response of the FoxO signaling pathway (hsa04068, Supplementary Figure 3A). These enhanced pathway responses included the cell cycle and DNA repair (CDKN1A, GADD45A, GADD45B, GADD45G), glycolysis (PCK2) and muscle atrophy (FBXO32) among others, while apoptosis was reduced (TNFSF10).

In addition, several significantly upregulated transcripts in non-healthy harbor porpoises were involved in the HIF1 signaling pathway which modulates responses to oxygen deficit (IL6R, INSR, IGF1R, AKT2, MKNK2, EIF4EBP1, PRKCA, CAMK2B, HMOX1, HK2, PFKFB2, CDKN1A; I04066, Supplementary Figure 3B). Transcripts implicated in detoxification reactions via glutathione were also found with higher expression profiles in non-healthy harbor porpoise muscles (CHAC1, GPX3, GLUL, GSTT3, CTH, Supplementary Table 3).

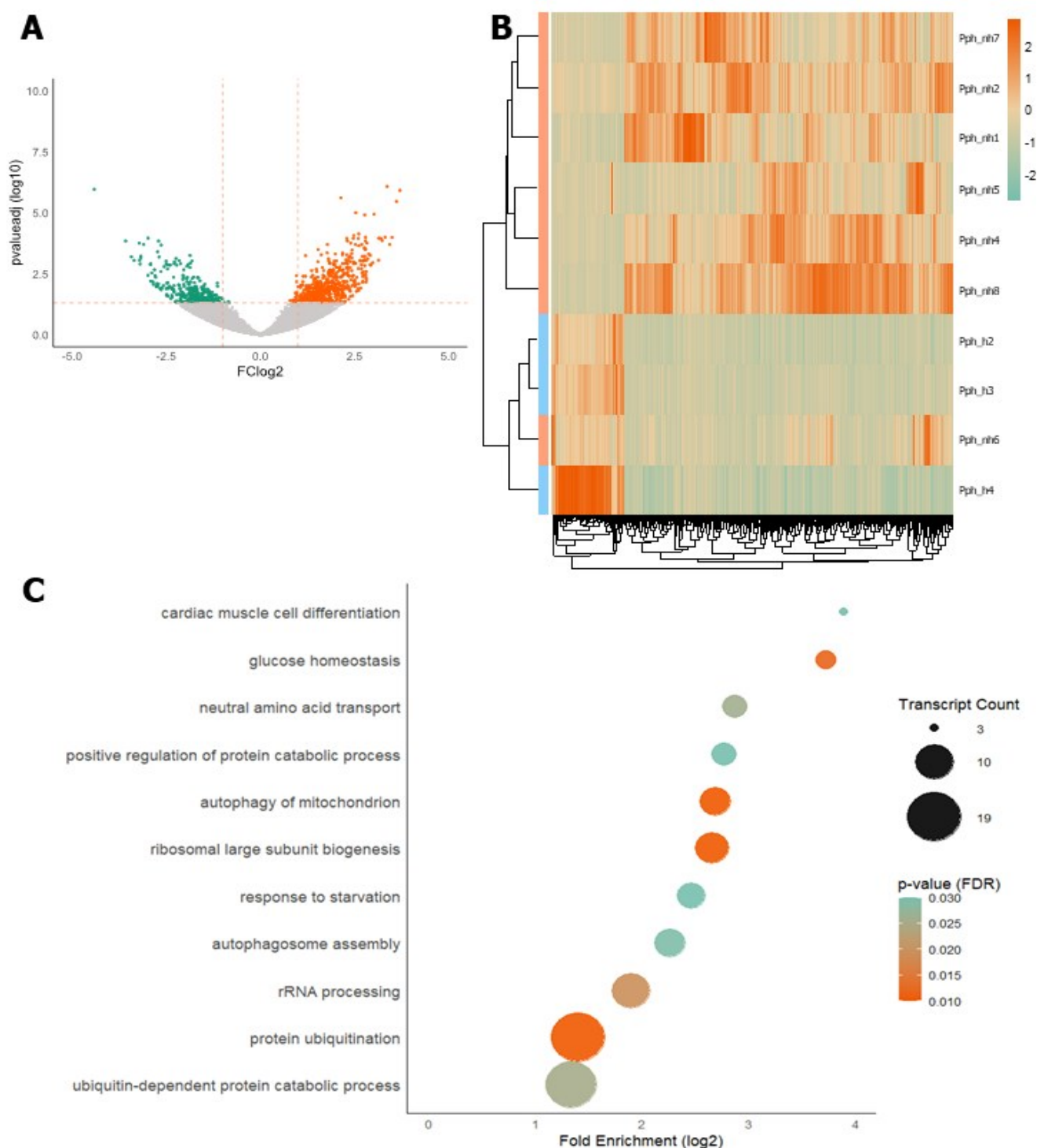


Figure 2 Differential analysis of the muscle. **A** Volcano plot of the significantly dysregulated transcripts in the muscle of non-healthy compared to healthy harbor porpoises. Significantly upregulated transcripts are highlighted in orange and downregulated transcripts in green. The x-axis shows the FC_{log2} and the y-axis depicts the FDR-corrected p-value (log_{10} -transformed). The applied cutoffs are highlighted by dashed orange lines ($FC_{log2} \geq 1/\leq -1$ and $pval_{FDR} \leq 0.05$). Transcripts were considered significantly dysregulated when $pval_{FDR} \leq 0.05$, $FC_{log2} \geq 1/\leq -1$ and mean $TPM_{non-healthy} \geq 5$. **B** Hierarchical clustering of the significant DETs in the muscle of non-healthy (apricot) and healthy (light blue) harbor porpoises. Upregulated transcripts are highlighted in orange and downregulated transcripts in green (see legend for FC_{log2} values). **C** Overrepresentation analysis of GO-Slim terms in “Biological Process” with PANTHER (reference: human) showed significant enrichment for upregulated transcripts, but not for downregulated transcripts. The y-axis shows the log_2 -transformed fold enrichment. Size of the dot represents the number of identified transcripts and the color of the dots indicates the significance level from high (orange) to low (green).

Multiple DETs within a single gene family exhibited concordant expression, suggesting coordinated family level regulation (Supplementary Table 3). This included the Kelch like gene family (6 DETs), solute carrier family (16 DETs), transmembrane-associated TMEMs (7 DETs) and of two transcription factor gene families (3 Maf transcription factors and 9 zinc finger transcription factors).

For the muscle, no significantly decreased pathways were identified with the GO and KEGG analysis. Collagen transcripts (COL1A2, COL3A1, COL1A1, Table 3), cell structure- and adhesion-associated DETs (e.g., CAPN6, FN1, NID2, MARCKS, FAP, ITGA2, CD38, TSPAN12; Table 3) were among the most significantly decreased transcripts in relation to healthy animals.

Table 3 Top 20 of the significant DETs in the muscles of non-healthy harbor porpoises.

Gene ID	Gene name	FC _{log2}	pval _{FDR}	Function (GeneCards/UniProt)
upregulated				
AMPD3	Adenosine Monophosphate Deaminase 3	3.71	1.22×10^{-06}	branch point in the adenylate catabolic pathway, muscular isoform
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A	3.62	3.45×10^{-06}	mediator of stress, responsive cell cycle G1 phase arrest
GADD45G	Growth Arrest And DNA Damage Inducible Gamma	3.50	1.03×10^{-04}	environmental stress response
TRIM63	Tripartite Motif Containing 63	3.44	2.00×10^{-04}	E3 ubiquitin ligase, muscle atrophy
KLHL30	Kelch Like Family Member 30	3.37	8.38×10^{-07}	may be involved in proteasome-mediated ubiquitin-dependent processes
KCNIP2	Potassium Voltage-Gated Channel Interacting Protein 2	3.36	1.47×10^{-25}	calcium-binding modulator of channel density and inactivation kinetics
H1-2	H1.2 Linker Histone, Cluster Member	3.24	1.38×10^{-04}	nucleosome structure and organization of chromatin
TNFRSF12A	TNF Receptor Superfamily Member 12A	3.21	1.13×10^{-04}	extrinsic apoptotic signaling pathway and wound healing
ANKRD33B	Ankyrin Repeat Domain 33B	3.15	1.10×10^{-04}	unknown, associated with muscle cells
LOC136143276 (IGHG4)	Immunoglobulin Heavy Constant Gamma 4	3.14	0.001	immune response, antibacterial humoral response and complement activation
IL6R	Interleukin 6 Receptor	3.02	1.13×10^{-05}	immune response, cell growth and differentiation regulation
CABYR	Calcium Binding Tyrosine Phosphorylation Regulated	2.94	0.001	calcium-binding
MAFF	MAF BZIP Transcription Factor F	2.92	0.001	dimeric transcriptional repressor, potentially involved in cellular stress response
IP6K3	Inositol Hexakisphosphate Kinase 3	2.92	0.001	converts inositol hexakisphosphate to diphosphoinositol pentakisphosphate
PRODH	Proline Dehydrogenase 1	2.88	4.86×10^{-04}	initial step in proline degradation
SDC4	Syndecan 4	2.85	0.001	intracellular signaling receptor involved in exosome biogenesis

IRS2	Insulin Receptor Substrate 2	2.83	1.76×10^{-04}	multifunctional signal transduction adapter
S100A12	S100 Calcium Binding Protein A12	2.82	0.006	inflammation and immune response
LYPD3	LY6/PLAUR Domain Containing 3	2.81	0.003	may enable laminin binding activity
PFKFB3	6-Phosphofructo-2 Kinase /Fructose-2,6-Biphosphatase 3	2.81	0.001	glycolysis regulation
downregulated				
COL1A2	Collagen Type I Alpha 2 Chain	-2.91	0.001	fibril-forming collagen
COL3A1	Collagen Type III Alpha 1 Chain	-2.73	3.97×10^{-04}	fibril-forming collagen
ITM2A	Integral Membrane Protein 2A	-2.69	0.001	may be involved in osteo- and chondrogenic differentiation
CAPN6	Calpain 6	-2.50	0.001	calcium-dependent cysteine protease responsive to oxidative stress
COL1A1	Collagen Type I Alpha 1 Chain	-2.41	0.010	fibril-forming collagen
G0S2	G0/G1 Switch 2	-2.39	0.001	extrinsic apoptotic signaling pathway
FN1	Fibronectin 1	-2.30	0.004	cell adhesion and migration, soluble dimeric and anchored multimeric form
TMEFF2	Transmembrane Protein With EGF Like And Two Follistatin Like Domains 2	-2.28	0.001	transmembrane protein
MEST	Mesoderm Specific Transcript	-2.20	0.047	possible role in development
OLFML1	Olfactomedin Like 1	-2.15	0.001	may be involved in signal transduction
NID2	Nidogen 2	-2.12	0.001	collagen-binding, basement membrane structure maintenance
MARCKS	Myristoylated Alanine Rich Protein Kinase C Substrate	-2.03	0.020	motility- and phagocytosis-modulating protein kinase C substrate
CALML4	Calmodulin Like 4	-2.03	0.002	myosin head/neck binding
RXRG	Retinoid X Receptor Gamma	-2.03	0.009	antiproliferative retinoic acid receptor
LXN	Latexin	-2.02	0.011	only known zinc-dependent metallocarboxypeptidase protein inhibitor
FAP	Fibroblast Activation Protein Alpha	-2.01	0.024	extracellular matrix degradation, tissue remodeling, wound healing and inflammation
ITGA6	Integrin Subunit Alpha 6	-1.96	0.005	cell surface adhesion and signaling
F2R	Coagulation Factor II Thrombin Receptor	-1.95	0.013	regulation of thrombotic response and of proinflammatory cytokines
CD38	CD38 Molecule	-1.94	0.024	messenger for intracellular calcium activation and glucose-induced insulin secretion
TSPAN12	Tetraspanin 12	-1.92	0.001	cell surface receptor, regulator of membrane proteinases

Shown are the gene ID and gene name, $FC_{\log 2}$, $pval_{FDR}$ and a short description of the main function (cut-offs for consideration: $pval_{FDR} \leq 0.05$, $FC_{\log 2} \leq -1$ and mean $TPM_{non-healthy} \geq 5$). Transcripts identified with a locus ID (LOC) are given in the brackets and the associated gene ID inferred from the NCBI Gene and UniProt databases. UniProt and GeneCards were used to define the main function.

Seven transcripts show similar high dysregulation in the lung and muscle

Comparing the significantly differentially expressed transcripts of the lungs to those of the muscles in non-healthy harbor porpoises with healthy harbor porpoises, we identified an overlap of seven dysregulated transcripts (Figure 3). Five DETs were found upregulated and two downregulated in both tissues, with a total of four DETs also found among the 20 most dysregulated transcripts (up: SBSN, S100A12; down: COL3A1, MFAP2; Tables 2, 3). Further concordant DETs were found in proteasome-associated DERL3 and stress mitigating GADD45B and ZBTB16.

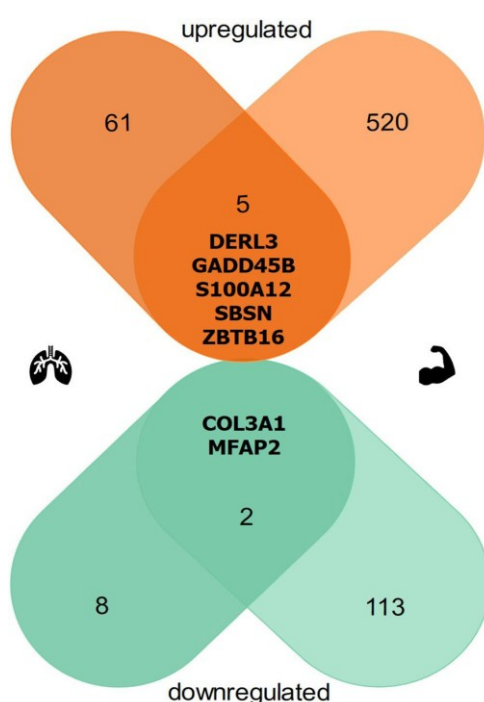


Figure 3 Venn diagram of simultaneously dysregulated transcripts in the lung and muscle of non-healthy harbor porpoises. In non-healthy harbor porpoises, five transcripts were significantly upregulated in the lung and muscle (DERL3, GADD45G, S100A12, SBSN, ZBTB16, depicted in orange), while two transcripts showed significant downregulation overlapping in both tissues (COL3A1, MFAP2, highlighted in green). 61 upregulated transcripts and eight downregulated transcripts were found only in the lung and 520 upregulated transcripts and 113 downregulated transcripts were identified only in the muscle.

De novo assembly shows dysregulation of several uncharacterized ncRNAs

To identify species-specific, potentially novel transcripts, we back-mapped the lung transcriptomes as well as the corresponding muscle transcriptomes to the *de novo* transcriptome assembly. The *de novo* assembly was generated using transcriptomes of four tissues (lung, muscle, brain, liver). A total of 204,013 Trinity transcripts were assembled, of which 148,936 were annotated with the Trinotate pipeline, using Blast and Diamond. Statistics of the *de novo*

assembly are given in Table 4. We employed strict cutoffs for the differential expression analysis ($p_{\text{valFDR}} \leq 0.05$, $FC_{\log 2} \geq 1$ or ≤ -1 , $\text{mean TPM}_{\text{non-healthy}} \geq 5$) and only considered Trinity transcripts that showed a $\text{TPM} \geq 0$ in at least half of the individuals in each condition group (healthy, non-healthy). This resulted in 24 significantly upregulated and 9 downregulated Trinity transcripts in the lung (Supplementary Table 4). In the muscle, 390 upregulated and 50 downregulated Trinity transcripts were identified (Supplementary Table 5). We identified several potentially novel transcript variants, lncRNAs and ncRNAs in the lung and muscle (Supplementary Tables 4, 5).

Table 4 Statistics of the *de novo* assembly.

Trinity statistics		
Total trinity transcripts	204,013	
Total trinity genes	129,859	
GC content	47.87	
	All transcript contigs	Longest isoform per gene
Total assembled bases	359,234,987	167,863,701
Mean contig length	1,760.84	1,292.66
Median contig length	1,057	802
N50	2,719	1,602
BUSCO		
	Cetartiodactyla	Mammalia
Complete	76.50%	78.00%
Duplicated	53.10%	55.10%
Fragmented	4.40%	5.60%
Missing	19.10%	16.40%

BUSCO, Benchmarking Universal Single-Copy Orthologs (highly conserved gene orthologs, expected to be expressed in all Cetartiodactyla/Mammalia).

For the *de novo* assembly, the Trinity and Trinotate pipeline was used. General statistics of the assembly are shown (total trinity transcripts, total trinity genes, GC content, annotated transcripts) and the pre-assembly quality was analyzed with the statistics of the mean contig length, median contig length and N50 for all transcripts and the longest isoform per gene. Post-assembly quality was inferred from identified orthologs in Cetartiodactyla and Mammalia using BUSCO.

In the lung, one downregulated Trinity transcript in non-healthy harbor porpoises was identified as ncRNA (LOC112408907), while in the muscle, a total of nine Trinity transcripts were identified as ncRNAs or lncRNAs (LOC116758475, LOC114484449, LOC137228934, LOC137231800, LOC109549532, LOC109549603, LOC117309420, LOC141276952, LOC116665024; Supplementary Table 5). Two upregulated Trinity transcripts of the muscle were determined to be an uncharacterized cetacean mRNA or protein (LOC118894103, LOC138842886; Supplementary Table 5). Lastly, the differential *de novo* assembly analysis in the muscle revealed four upregulated and seven downregulated Trinity transcripts that could

not be further identified by Blast, Diamond or NCBI Blastn/Blastp with the applied cut-offs (Supplementary Table 5).

Discussion

Harbor porpoise populations inhabiting the North and Baltic Seas are experiencing increasing impacts due to a variety of human activities, including underwater-radiated noise and chemical pollution. This can have adverse effects on immunocompetence, resulting in an increased susceptibility to pathogens and infectious diseases. Harbor porpoises suffer from parasitic and bacterial infections of the respiratory tract and associated lesions which may impair the lung function. Since harbor porpoises develop respiratory lesions to a severity which is not found in humans and terrestrial animals, it is not known how they deal with effects on reduced oxygen uptake as diving mammals. As an air-breathing, fully aquatic mammal and apex predator, this may have a negative effect on their viability, if receiving organs, such as the skeletal muscles, are supplied with insufficient oxygen, as they consume enormous amounts of oxygen and energy to enable diving ability and foraging.

Broad activation of immune and surfactant processes and ECM-modulating transcripts indicate effort to mitigate pathogenic insults

Harbor porpoises suffering from severe nematode infestations and associated bacterial bronchopneumonia exhibited an expected, pronounced immune response in the lung tissue. As one of the first interfaces of the organism with its environment, the respiratory tract functions as a primary responding tissue to detrimental viral, bacterial and pathogenic insults, thus employs a wide array of innate and adaptive immune responses (Galeas-Pena et al., 2019). The high expression of immunoglobulins and leukocytes suggests increased opsonization of pathogens (Chauhan et al., 2024), while inflammatory cytokines, such as CXCL8 and CXCL17, indicate an acute activation of adaptive immune responses in the lungs of non-healthy harbor porpoises (Carvallo & Stevenson, 2022).

Large nematodes within the tissue not only disrupt tissue integrity, but also cause inflammation and can facilitate bacterial or viral infection. In mammals, this typically induces a T helper cell type 2 (Th2) immune response to manage nematode load and tissue regeneration (Allen & Maizels, 2011). While we observed some upregulation of genes in the lungs of non-healthy

harbor porpoises that are also implicated in the Th2 immune response, such as IGHG1, MMP3, MMP9 and TIMP1 (Allen & Maizels, 2011; Carvallo & Stevenson, 2022), our results do not suggest a definite activation of the Th2 response. This is consistent with a recent study which observed a Th2 immune response in harbor porpoises suffering from mild to moderate lungworm burden, but not in severe cases (Tsang et al., 2025). Since our distinction between non-healthy and healthy porpoises was solely based on severity of nematode burden in the respiratory tract, our non-healthy animals likely represent severe disease cases. Whether this is caused by overwhelming and prolonged nematode burden or exacerbating immune challenge and subsequent compromise remains to be elucidated.

To enable influx of recruited immunocytes and other cell types necessary for wound healing to the inflamed or infested area, matrix metalloproteinases readily degrade the extracellular matrix and its components (Madri & Graesser, 2000). This turnover of the ECM can be indicated by the ratio of MMPs to TIMP1, their inhibitor, with increased MMPs resulting in higher degradation rates. Here, non-healthy harbor porpoises appear to show higher ECM degradation, as indicated by higher upregulation of MMP3 and MMP9 compared to TIMP1 and dysregulation of ECM processes. Although tissue degradation is also an important step to enable tissue remodeling and regeneration, non-healthy harbor porpoises showed a marked decrease in expression of type III collagen, a main constituent of healthy lungs (Burgess & Weckmann, 2012). It is therefore plausible that harbor porpoises with exceeding nematode burden and poor pulmonary health may struggle to adequately and swiftly replace damaged or degraded tissue which can lead to accelerated permanent tissue loss and functional impairment.

Within the respiratory system, the surfactant mucus lining facilitates respiration, but also represents an additional layer of immune protection (Wright, 2005). Likewise, lungs of non-healthy harbor porpoises displayed higher expression of surfactant transcripts with a primary role in host defense and airway clearance, and respiration and hypertension relief (Foot et al., 2006; Wright, 2005). Surfactant is composed of ~85% phospholipids and has a high turnover rate in healthy lung tissue that can be accelerated or inhibited in compromised lungs (Milad & Morissette, 2021). However, disrupted surfactant production can have negative consequences if recycling capacity is exceeded and can manifest itself in the form of atherosclerosis-like symptoms (Gurel et al., 2001; Moore et al., 2013). Disease-associated surfactant alterations in lipid composition have also been found to affect lung structure and mechanical function in dolphins (Porrás-Gómez et al., 2025). In line with this, lungs of non-healthy harbor porpoises

exhibited upregulation of several transcripts involved in lipid-associated atherosclerosis, hinting at pathologically altered regulation.

Often considered as a passive diffusion organ, the lungs are metabolically active (Liu & Summer, 2019). Despite being one of the most well-oxygenated tissues, the lungs largely utilize lactate as energy substrate for aerobic glycolysis, potentially to reduce oxygen consumption and preferably distribute oxygen to distant organs (Fisher, 1984). Here, no transcripts were significantly dysregulated that indicated higher lactate conversion. However, the observed elevation of carbohydrate transport suggests an increased cellular glucose metabolism, as host defense, surfactant production and respiration are energy-intensive processes (Fisher, 1984; Liu & Summer, 2019). This is in line with previous studies in other mammals which showed that the lung glucose consumption in part exceeds that of other highly metabolic organs (Fisher, 1984; Liu & Summer, 2019).

Enhancement of HIF1 and stress-responsive signaling, immune activation and cell cycle checkpoints point to downstream effect of lung lesions in the skeletal muscles

The comparative muscle transcriptome analysis indicates an effect from the oxygen-uptaking lung to the oxygen-demanding muscle of harbor porpoises with compromised respiratory health. Muscles exhibited enhanced stress signaling pathways and transcripts that pointed to elevated endurance of cumulative stressors, including hypoxia, starvation, chemical stress and inflammation, respectively. Enrichment of the key hypoxia-mediating HIF1 signaling pathway as well as of the stress-responsive FoxO signaling pathway implicates that these possibly are important signaling pathways involved in the muscle response of harbor porpoises with a compromised lung health. Here, HIF1-regulated transcripts were significantly elevated in non-healthy porpoises that shift energy production to glycolysis, a hallmark feature of hypoxic and inflammatory states and which is utilized by cetacean muscles to fuel diving under depleting oxygen availability (Castellini et al., 1981; Kierans & Taylor, 2024; Kooyman et al., 1980a). The muscle is highly metabolic, serves as nutrient and oxygen storage in whales, but also consumes enormous amounts of energy to fuel energetically-costly diving and an aquatic lifestyle (Davis, 2014; Pabst, 1993). Additionally, harbor porpoises are known to have a high metabolic field rate, resulting in constant foraging (Rojano-Donãte et al., 2018), but do not possess large energy reserves due to their relatively small size and muscle mass, putting them at higher risk of starvation (Spitz et al., 2012).

Indicating similarities to studies of patients with lung diseases such as COPD (Henrot et al., 2023; Plant et al., 2010; Puig-Vilanova et al., 2015), the muscles of non-healthy harbor porpoises displayed enrichment of autophagy and muscle atrophy pathways. We also observed reduced cell integrity which can be exacerbated by both pulmonary disease and insufficient oxygen (Henrot et al., 2023; Puig-Vilanova et al., 2015; Rom & Reznick, 2016). While autophagy is a normal process in the healthy musculature that enables regeneration and muscle growth, under metabolic deprivation or stress, disproportionate regulation leads to utilization of muscle protein to fuel essential functions (Call & Nichenko, 2020; Whiteman et al., 2017). This may further aggravate this incompetence in harbor porpoises (Rojano-Donāte et al., 2018; Wisniewska et al., 2018) and moreover, disables harbor porpoises to avoid other stressors, such as underwater-radiated noise which causes spontaneous deeper diving or rapid swim speed acceleration (Elmegaard et al., 2023; Frankish et al., 2023). In accordance with our previously published results (Dönmez et al., 2023), protein synthesis and regeneration-associated transcripts were elevated, while muscles seemingly also employed mitotic cell cycle checkpoints. This coordinated response may aid in minimizing DNA damage and reduces accumulation of defective cells which is important since muscles are a post-mitotic tissue that needs to adapt quickly to environmental changes (Call & Nichenko, 2020; Frontera & Ochala, 2015; Howard et al., 2020).

While the muscle of non-healthy harbor porpoises showed elevated anaerobic metabolism and higher atrophic states, the transcriptomic analysis hinted at a decreased leak respiration and more efficient energy utilization by lower expression of uncoupling protein UCP3 ($FC_{\log 2} = -2.04$; $p_{\text{valFDR}} = 0.069$) and elevation of its regulator NPY ($FC_{\log 2} = 2.04$; $p_{\text{valFDR}} = 0.035$). UCP3 has not yet been detected in cetaceans. Studies have described its pseudogenized paralog UCP1 in cetaceans and postulated a function in lipid processes (Gaudry et al., 2017; Zhou et al., 2022), however the integrity and function of cetacean UCP3 remains to be elucidated.

Cross-tissue dysregulated transcripts are affected by environmental stressors

Our systemic transcriptome analysis revealed five upregulated and two downregulated DETs in the lungs and muscles that were simultaneously dysregulated in non-healthy harbor porpoises. The concordant regulation of this DET subset in both tissues highlights their potential role in the extrapulmonary, pathogenic processes extending beyond the lungs. This included upregulated SBSN which has been associated with hypoxic environments (Goyal & Longo, 2014). SBSN was also found elevated after endurance exercise in sled dog muscles (Brass et

al., 2009), suggesting a causal link between prolonged muscle exercise capacity and inadequate oxygen concentration. Further supporting a connected systemic response to an impaired lung function, we observed a significant upregulation of stress signaling transcripts GADD45G and ZBTB16. While GADD45G is swiftly upregulated upon genotoxic and environmental stress (Liebermann & Hoffman, 2008), ZBTB16 is a transcription factor in response to cold stress and modulates higher utilization of fatty acid metabolism and glycolytic capacity (Plaisier et al., 2012). Elevated serum levels of S100A12 are indicative of the inflammatory progression of acute and chronic lung diseases (Lorenz et al., 2008), while in skeletal muscle it is not predominantly found or expressed. Hence, the upregulation in the lung and muscle of non-healthy harbor porpoises may stem from the compromised respiratory system and contribute to multi-organ dysfunction in the muscle. The systemic dysregulation of structural transcripts (COL3A1, MFAP2) and of transcripts associated with the ubiquitin-dependent proteasome (DERL3) points to limited tissue remodeling and ECM structure (Belmont et al., 2010; McCabe et al., 2023), which is consistent with observations in other studies of extrapulmonary morbidities of respiratory diseases (Barreiro & Jaitovich, 2018; Plant et al., 2010; Puig-Vilanova et al., 2015).

However, the lungs and the skeletal muscles are highly vascularized tissues. Thus, the question remains as to what extent the simultaneous dysregulation of these DETs is attributable to the blood circulation from other organs, since non-healthy harbor porpoises suffered from several diseases, including lesions in the pulmonary blood vessels. Although we cannot be certain that the observed regulation is exclusively driven by the pathological lung lesions, the concordant dysregulation of several transcripts highlights how disease states induce a coordinated, systemic injury response in harbor porpoises.

***De novo* assembly reveals several potentially regulatory ncRNAs in the lung and muscle**

The *de novo* assembly of the harbor porpoise lung and the muscle displayed overall similarly dysregulated biological processes in non-healthy harbor porpoises like the differential analysis (data not shown). However, several significantly dysregulated Trinity transcripts encoded for non-coding (nc) RNAs, which may possess an important role in rapid immunoregulation and time-sensitive responses to environmental or DNA-damaging stimuli. Similar observations have been stated in studies of humans and mice, which found ncRNAs to have distinct spatiotemporal expression profiles that can rapidly change upon external insults and disease to

alter immune cell composition and responses (Grammatikakis & Lal, 2022; Mowel et al., 2018). Therefore, the function and effect of the dysregulated ncRNAs in the lungs and muscles of non-healthy harbor porpoises should be subject of future *in vitro* studies, since they could have a profound immunoregulatory effect in response to the observed lung lesions and systemic reaction from the muscles. Two significantly upregulated Trinity transcripts in the lung of non-healthy harbor porpoises originated from pathogenic helminths (data not shown), emphasizing the severity of parasite accumulation in non-healthy harbor porpoises (Reckendorf et al., 2021). Furthermore, several Trinity transcripts that were significantly upregulated in the muscles of non-healthy harbor porpoises did not have a definite or unique match with other orthologs when analyzed with Blast, Diamond and NCBI Blast (as per our definition and cut-offs), thus potentially describe novel transcript variants. However, methods used for *de novo* assemblies and RNA-Sequencing can lead to an overestimate of identified transcripts. While we employed strict cut-offs and applied necessary precautionary filtering of the results to reduce such inconvenient effects, it is still possible that some of these variants may stem from overestimation or transcriptional noise amplified by the assembly. Another restricting factor is that reference databases for non-model organisms, such as marine mammals, are often incomplete and with substantial blanks which is due to the challenges of access to the animal, sampling methods and experimental limitations. Therefore, the definite classification of these putative transcripts and their function have to be studied in greater detail and be verified in further molecular studies.

Limitations

The samples used in this study are from necropsies of wild harbor porpoises that were dead for under 24 hours. Lungs act as vital interface between the animal and its environment, thus are constantly exposed to external impacts which might result in a more rapid degradation of the lungs compared to other tissues (e.g., muscle). Also, the observed pathological lesions in the respiratory tract and lungs may further accelerate tissue degeneration. In addition to decomposition state, access to marine mammal samples is generally very limited due to their aquatic, elusive lifestyle and conservation status (Van Cise et al., 2024). Within our two cohorts, there is an age class difference that overlaps with health status. This is in part also caused by high percentage of young harbor porpoises among post-mortem examined individuals and that adult harbor porpoises are rarely in good pulmonary health (Siebert et al., 2001, 2020). Harbor porpoises accumulate parasitic and pathogenic load with increasing food intake and advancing age (Jepson et al., 2005; Sonne et al., 2020a). Therefore, it cannot be entirely ruled out that age

class may affect gene expression. The observed interindividual variation may also stem from differences in age, disease, and not known impacts prior to death which might result in diverse molecular signatures. Here, one juvenile individual classified as non-healthy (Pph_nh6, approx. one year old) groups with the healthy cohort in the comparative analysis of the muscle. Calves develop their musculature rapidly within the first year (Noren et al., 2014), while suffering from comparably less nematode presence than adults. This may result in downstream impacts that are not yet as severe as those in affected adults.

Conclusion

Harbor porpoises of the North and Baltic Seas increasingly suffer from pathological lesions in the lungs, that are caused and aggravated by accumulating parasitic load and associated secondary infections. These pathological lesions may disrupt lung tissue function, reducing oxygen uptake and supply to distant, receiving organs. This study provides deeper insights into the underlying compensatory and pathophysiological mechanisms of the lungs and locomotor muscles of harbor porpoises with a compromised pulmonary health. Our transcriptome analysis showed similar immune responses and defense mechanisms compared to humans suffering from lung diseases, suggesting that these reactions are conserved in mammals regardless of their distinct environments. Although nematode infections typically trigger a Th2 immune response, the lung transcriptomes did not show such expression profiles, which is consistent with previous observations of a reduced Th2 activation in harbor porpoise lungs with severe nematode infestation. Lungs of non-healthy harbor porpoises displayed higher expression of surfactant transcripts that mainly aid with respiration, and enhanced carbohydrate transport which may help to replenish diminishing energy reserves. Our results suggest a detectable systemic effect of the compromised lung on the locomotor muscles, resulting in a response to multiple impacts, such as inflammation, hypoxia and starvation. Such effects on the muscles have also been observed on humans suffering from lung diseases. A swift, but tightly regulated switch between regeneration and degradation in the muscles may represent an attempt to alleviate excessive muscle atrophy to uphold tissue function. However, mitigating cumulative impacts is strenuous and requires substantial amounts of energy which may be challenging for non-healthy harbor porpoises. The *de novo* assembly revealed several highly dysregulated non-coding RNAs in the lungs and muscles of non-healthy harbor porpoises. Non-coding RNAs possess important regulatory functions, thus may cause fast spatiotemporal responses to distinct

stimuli. Although some molecular immune responses in cetaceans are not well understood, this study provides relevant insights into cetacean immune responses to diseases and pathogens and their interaction with anthropogenic stressors, thereby supporting conservation efforts.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/>, PRJNA977857.

Ethics statement

Ethical review and approval were not required for the study since the investigated animals were collected once they were dead by stranding or by-caught within the German stranding network. The German stranding network conducts work such as collecting and holding carcasses and samples from European protected species following appropriate licenses from the relevant authorities.

Author contributions

ED: Writing – original draft, Formal analysis, Investigation, Data curation, Project administration, Conceptualization, Methodology, Visualization, Validation. AF: Conceptualization, Project administration, Supervision, Writing – review & editing, Funding acquisition. US: Writing – review & editing, Funding acquisition, Conceptualization, Resources, Supervision, Data curation.

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Conflict of interest

The authors declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author US declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2026.1738836/full#supplementary-material>

7 Chapter III

From the lung to the muscle – Insights from an integrative MultiOmics analysis of harbour porpoises in poor respiratory health

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Abstract

Harbour porpoises (*Phocoena phocoena*) in the North and Baltic Seas are increasingly impacted by anthropogenic pressures, including underwater noise, fisheries and pollution. These pressures correlate with declining population health, particularly affecting the respiratory system. Growing pathological lesions, partly resulting from high prevalence of parasitic infestations and subsequent diseases, can impair tissue function and oxygen supply to distant end-organs.

In this study, we applied an integrative MultiOmics approach (proteomics, metabolomics, lipidomics) to analyse the lungs and muscles of 12 wild harbour porpoises with compromised respiratory health. Our aim was to identify dysregulated biological pathways across omics layers to advance insights into adaptive physiological responses and to define disease-associated molecular signatures that could assist health assessments.

Our analysis revealed pronounced immune system and antioxidative responses in the lungs and muscles, indicated by enhanced immunoglobulins, plasmalogens and glutathione-related proteins. In the lungs, high cardiolipin levels and reduced collagen suggest impaired tissue structure and function, while tissue maintenance processes were elevated in the muscle. Both tissues exhibited metabolic alterations suggestive of energetic imbalance, including increased purine metabolism in the lung and decreased lipid metabolism in the muscle. Several dysregulated molecules were shared across tissues, pointing to pathophysiological effects. The proposed disease-associated molecular signatures included the protein SLC25A4, the metabolite O-phosphoethanolamine and the lipid TG O-16:0_16:0_20:4 for the lung, and the protein SPEG, the metabolite pipercolic acid, and the lipid BMP 18:1_22:6 in the muscle.

Our findings elucidate the complexity of molecular mechanisms linking anthropogenic and environmental stressors with vulnerability and resilience in a marine sentinel species. Furthermore, this study highlights the potential of integrative omics to define disease-related marker panels, thereby supporting ongoing and future health monitoring and conservation efforts.

KEYWORDS harbour porpoise, oxidative stress, proteomics, metabolomics, integrative MultiOmics, molecular toolbox, marine mammals

Introduction

The harbour porpoise (*Phocoena phocoena*) is a cetacean abundant across the North Atlantic and frequently encountered in coastal areas. In the North and Baltic Seas, the harbour porpoise also functions as a sentinel species indicating ecosystem status, and several distinct subpopulations with differing habitats and conservation concerns exist (Carlström et al., 2023; Koschinski et al., 2024; Nachtsheim et al., 2021; Owen et al., 2024). As economic interests in these waters rise, their impact on cetaceans results in cumulative stress which can reduce fitness and cause higher morbidity (Carlén et al., 2021; Kesselring et al., 2017; Siebert et al., 1999; Vinther & Larsen, 2004; Wisniewska et al., 2018). Although many natural and anthropogenic pressures affect harbour porpoises, underwater-radiated noise is a growing concern as dependence on maritime infrastructure and construction continuously increases (Halpern et al., 2008). With several offshore constructions and important harbours located in German waters, noise pollution accumulates and interferes with the porpoises' communication, hearing and behaviour (Erbe et al., 2019; Kastelein et al., 2018). Free-ranging individuals display a profound flight-and-avoidance reaction when encountering ships or acoustic deterrence signals, including spontaneous, for the species atypical prolonged and deeper dives (Elmegaard et al., 2023; Frankish et al., 2023; Wisniewska et al., 2018). While harbour porpoises generally perform short dives within 30-50 m, as the North and Baltic Seas are mostly shallow waters (Teilmann et al., 2007; Westgate et al., 1995), tagged porpoises were able to perform deeper dives, if the bathymetry allowed such dive profiles (Teilmann et al., 2007).

Diving demands large amounts of energy for the muscles to fuel force and speed, for which adequate oxygen supply is necessary. To enable diving bouts, marine mammal muscles possess higher myoglobin concentrations to store oxygen (Arregui et al., 2021), and are more tolerant of anaerobic metabolism to sustain energy levels upon oxygen depletion (Castellini et al., 1981). In addition to the blood and muscles, harbour porpoises also use their lungs for oxygen storage (Piscitelli et al., 2010). However, overall health and especially the pulmonary health of harbour porpoise populations is declining (Ryeng et al., 2022; Siebert et al., 2001, 2009). Their lungs are commonly found with infestations of nematodes that can cause local inflammation, obstruction of blood vessels and increase the susceptibility to additional diseases (IJsseldijk et al., 2021; Jepson et al., 2000; Siebert et al., 2001, 2006a). Aberrant pathological lesions may not only obstruct the capacity of the lung to take up sufficient oxygen, but may also affect distant, oxygen-dependent organs, such as the muscles (Ceco et al., 2017; Siebert et al., 2001; Ten Doeschate et al., 2017). Inadequate oxygen supply to the locomotor muscles may result in

impaired diving ability, if the disparity between muscular oxygen demand and supply by the lung is too large to compensate. In captive harbour porpoises, it has been observed that they reach only ~60% of the complete lung capacity (Rojano-Donate et al., 2018). However, it has yet to be clarified, whether the reduced utilisation is due to tissue function impairment or if it represents a voluntary behaviour and harbour porpoises do not need to exploit their full lung capacity.

Although many adaptations of marine mammals have been extensively investigated (Allen & Vázquez-Medina, 2019; Davis, 2014; Hindle, 2020), some molecular and physiological mechanisms underlying diving in cetaceans remain unresolved. Studies on cetaceans are limited by access to samples due to their fully aquatic lifestyle and conservation status (Van Cise et al., 2024). Additionally, reference databases of non-model organisms, such as marine mammals, are not always available and complete for various tools (Cammen et al., 2016), hence hinder in-depth analyses. Omics studies offer an alternative to gain knowledge about environmental impacts and molecular adaptations, even if sample sizes are small and limited. In marine mammal science, omics are an emerging tool and have been mostly applied on cetacean biopsy or blood samples (Kershaw et al., 2018, 2024; Morey et al., 2022; Trego et al., 2019b; Veldhoen et al., 2012). Current studies often rely on one or two omics layers to analyse dysregulated biological processes and pathways (Deros et al., 2022; Houser et al., 2021; Simond et al., 2025; Trego et al., 2019b). Combining multiple omics methods provides holistic insights into systems biology and cross-layer regulation, particularly for inaccessible, non-model organisms (Mancia, 2018). Moreover, a deeper understanding of molecular and physiological responses to disease and stress in cetaceans is crucial to assess population resilience and vulnerability under intensifying environmental pressures (Lennon et al., 2025), thereby supporting long-term monitoring efforts.

Here, we performed comparative, untargeted MultiOmics including proteomics, metabolomics and lipidomics of the lungs and locomotor muscles of free-ranging harbour porpoises that died from stranding or as by-catch. We compared porpoises suffering from severe pathological lung lesions, partly due to nematode infestations and bronchopneumonia, against porpoises without lung-associated diseases to assess dysregulated biological pathways. For both tissues, we further analysed the correlation between the health status and dysregulated features to tentatively identify a lung disease-associated, molecular signature that may serve as an analytical tool to advance disease diagnostics and support conservation efforts.

Materials and Methods

Animals and Sampling

Tissue samples of the lung and muscle (*Musculus longissimus dorsalis*) were collected during routinely performed necropsies of harbour porpoises (n = 13, Tab. 1) at the Institute of Terrestrial and Aquatic Wildlife Research, University of Veterinary Medicine Hannover, Foundation, Büsum, Germany, which is part of the German stranding network (Benke et al., 1998; Siebert et al., 2006b). Harbour porpoises died by stranding or were by-caught between April 2016 and August 2022. Decomposition was not advanced (decomposition code II, estimated within 12-24 h of death). Full necropsies and further investigations of the animals were performed according to standardised protocols (IJsseldijk et al., 2019; Siebert et al., 2001). Depending on the necropsy findings, harbour porpoises were categorized as non-healthy or healthy. Harbour porpoises classified as non-healthy exhibited considerable pathological lesions due to nematode infestations (lungworms) and subsequent bacterial infections in the lung, and suffered or died from bronchopneumonia (Tab. 1). Healthy porpoises displayed minor to no pathological lesions in the lung and were in good overall health. Tissue samples were stored at -20 °C until further use.

Table 1 Metadata of the wild harbour porpoises used in this study. Health condition, sex, year of sampling, age class, habitat, bycatch, nutritional state and disease/cause of death are given. Non-healthy harbour porpoises were defined as animals that suffered from severe levels of lungworm infestation and bronchopneumonia. Healthy individuals did not suffer or die from lung-associated diseases or infestations. Nutritional state is defined as G: good, M: moderate, P: poor and u: undetermined. Animals were predominately from the Baltic Sea (BS) and three individuals were from the North Sea (NS).

Individual	Condition	Sex	Sampling Year	Age Class	Location	By Catch	Nutrition	Disease/Death
Pph_h1	healthy	male	2020	neonate	BS	no	u	Perinatal death
Pph_h2	healthy	male	2020	neonate	BS	suspected	u	None
Pph_h3	healthy	male	2021	juvenile	BS	yes	G	Mild inflammation in the lung, stomach and liver
Pph_h4	healthy	female	2022	neonate	NS	no	G	Perinatal death
Pph_h5	healthy	female	2016	juvenile	BS	yes	G	None
Pph_h6	healthy	male	2020	juvenile	BS	suspected	G	Indicators of shock
Pph_nh1	non-healthy	female	2019	adult	BS	suspected	M	Bronchopneumonia, hepatitis, adrenalitis, final septicaemia (<i>P. Multocida</i>)
Pph_nh2	non-healthy	male	2019	juvenile	NS	no	M	Bronchopneumonia, gastroenteritis
Pph_nh3	non-healthy	male	2022	juvenile	BS	yes	G	Bronchopneumonia, gastritis
Pph_nh4	non-healthy	female	2022	adult	NS	no	P	Meningoencephalitis, bronchitis, gastritis

Pph_nh5	non-healthy	male	2022	juvenile	BS	yes	G	Bronchopneumonia, gastritis, endoparasitosis
Pph_nh6	non-healthy	male	2021	juvenile	BS	yes	G	Bronchopneumonia, gastritis
Pph_nh12	non-healthy	female	2021	adult	BS	yes	G	Bronchopneumonia, endoparasitosis

LC-MS/MS-based bottom-up proteomics

Tryptic digest

For the proteomic analysis, 10 mg of frozen lung and muscle tissue were provided to the Core Facility Mass Spectrometric Proteomics of the University Medical Center Hamburg-Eppendorf.

Samples were dissolved in 100 mM triethyl ammonium bicarbonate and 1% w/v sodium deoxycholate buffer, boiled at 95 °C for 5 min and sonicated with a probe sonicator. The protein concentration of denatured proteins was determined with the Pierce™ BCA Protein assay kit (Thermo Fisher, USA), and samples were diluted to 20 µg protein in 50 µL buffer. Samples were dissolved to a concentration of 70% acetonitrile (ACN). Carboxylate-modified magnetic beads (1 µL, GE Healthcare Sera-Mag™, Chicago, USA) at 1:1 (hydrophilic/hydrophobic) ratio in methanol/LC-MS-grade water were added, following the SP3-protocol workflow (Hughes et al., 2019). Samples were shaken at 1400 rpm for 18 min at room temperature. Tubes were placed on a magnetic rack and supernatant was removed. Magnetic beads were washed two times with 100% ACN and 70% ethanol. After resuspension in 50 mM ammonium bicarbonate, disulfide bonds were reduced in 10 mM dithiothreitol for 30 min and alkylated in presence of 20 mM iodoacetamide for 30 min in the dark. Samples were digested with sequencing-grade trypsin (Promega, USA) at 1:100 (enzyme:protein) ratio at 37 °C and shaken at 1400 rpm overnight. Tryptic peptides were bound to the beads by adding 95% ACN and shaken at 1400 rpm for 10 min at room temperature. Placed on the magnetic rack, the supernatant was removed and the beads were washed twice with 100% ACN. Elution was performed with 2% DMSO in 1% formic acid (FA). The supernatant was dried in a vacuum centrifuge and stored at -20 °C until further use.

LC-MS/MS data acquisition

Chromatographic separation of peptides was achieved with a two-buffer system (buffer A: 0.1% FA in H₂O; buffer B: 0.1% FA in ACN) on a nano-Ultra High-Performance Liquid

Chromatography system (Dionex Ultimate 3000 UPLC with a NanoViper C18 peptide trap (5 μm , 100 μm x 20 mm), Thermo Fisher, USA) for online desalting and purification, followed by a nanoEase M/Z Peptide BEH C18 column (1.7 μm , 75 μm X 250 mm; Waters, USA). Peptides were separated using an 80 min-method with linearly increasing ACN concentration from 2% to 30% over 60 min. MS/MS measurements were performed on a quadrupole-orbitrap hybrid mass spectrometer (QExactive, Thermo Fisher, USA). Eluting peptides were ionized using a nano-electrospray ionization source with a spray voltage of 1800 and analysed in data-dependent acquisition mode. For each MS1 scan, ions were accumulated for a maximum of 240 ms or until a charge density of 1×10^6 ions (AGC target) was reached. Fourier-transformation-based mass analysis of the data from the orbitrap mass spectrometer was performed covering a mass range of 400 – 1200 m/z with a resolution of 70,000 at $m/z = 200$. Peptides, responsible for the 15 highest signal intensities per precursor scan with a minimum AGC target of 5×10^3 and charge state from +2 to +5, were isolated within a 2 m/z isolation window and fragmented with a normalised collision energy of 25% using higher energy collisional dissociation. MS2 scanning covered a mass range starting at 100 m/z and was accumulated for 50 ms or to an AGC target of 1×10^5 at a resolution of 17500 at $m/z = 200$. Already fragmented peptides were excluded for 20 s.

Data processing and quantification

LC-MS/MS data were searched with the Sequest algorithm, integrated into the Proteome Discoverer software (v 3.0; Thermo Fisher, USA), against a reviewed vaquita database (*Phocoena sinus*, TaxID=9741_and_subtaxonomies, 2023). Lung and muscle tissue samples were searched separately. Carbamidomethylation was set as a fixed modification for cysteine residues. The oxidation of methionine and pyro-glutamate formation at glutamine residues at the peptide N-terminus, as well as acetylation of the protein N-terminus were allowed as variable modifications. A maximum number of two missing tryptic cleavages was set. Peptides between 6 and 144 amino acids were considered. A strict cut-off (FDR < 0.01) was set for peptide and protein identification. Quantification was performed using the Minora algorithm, implemented in Proteome Discoverer, using the match-between-runs function.

Statistical analysis

Obtained protein abundances were log2-transformed and normalised by column-median normalisation. As the sample preparation was performed in two batches, batch effects between the two measurements were removed using the BERT algorithm (Schumann et al., 2025). In

total, 2560 proteins were identified in lung tissue and 1526 proteins in muscle tissue. The data was reduced to proteins found in at least 70% of all samples and a two-sided unpaired Student's T-test was performed using the statistic program Perseus (Tyanova et al., 2016), including permutation-based false discovery rate (FDR)-correction with 250 randomisations and q-value cut-off of 0.05.

One individual was not included in the subsequent analyses, since it was an outlier in multiple experiments. This may be due to advanced tissue degradation, possibly due to freeze-and-thawing before necropsy. Normalised data of the lung and muscle proteomes can be found in Supplementary Tab. 2-3.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD070379.

Metabolomics and Lipidomics extraction and identification

To perform untargeted metabolomics and lipidomics, ~50 mg of frozen lung and muscle tissue were supplied to the Metabolomics Core Facility of the European Molecular Biology Laboratory, Heidelberg, Germany.

Sample extraction was initiated by adjusting tissue concentration homogenously across samples to 75 mg/mL via addition of 80% methanol, including internal standards (final conc.: 0.5% and 1.0%; for metabolomic profiling of small, polar molecules: MSK-A2-1.2, Cambridge Isotope Laboratories, USA; for lipidomics: EquiSPLASH, Avanti Polar Lipids, USA). After homogenisation on dry ice with a bead beater (FastPrep-24; MP Biomedicals, CA, USA) at 6.0 m/s (5 x 30 s, 5 min pause) using 1.0 mm zirconia beads (Biospec Products, OK, USA), samples were incubated at -20 °C for 20 min. Subsequently, samples were vortexed and 187.5 µL per sample were transferred to a fresh 1.5 mL tube. For biphasic extraction of lipids and polar metabolites, 500 µL methyl tert-butyl ether were added. The monophasic mixture was vortexed for 60 s and incubated at -20 °C for 20 min. For phase separation, 87.5 µL water were added, followed by another vortex and incubation step (see previous conditions). The biphasic solvent system was then centrifuged for 10 min at 15,000g and 4 °C.

For lipidomics analysis, 400 µL of the upper organic phase were transferred, dried under a nitrogen stream and reconstituted in 200 µL isopropanol:methanol (50:50). For metabolomics analysis, 250 µL of the lower aqueous phase were transferred, dried under a nitrogen stream, and reconstituted in 100 µL ACN/methanol/H₂O (2:2:1). The final samples were vortexed for

10 min, centrifuged (see previous conditions) and the supernatants were transferred to analytical glass vials for LC-MS/MS analysis.

The LC-MS/MS analysis was performed on a Vanquish Horizon UHPLC system, coupled to an Orbitrap Exploris 240 high-resolution mass spectrometer (Thermo Scientific, MA, USA) in negative and positive electrospray ionisation mode.

Untargeted Lipidomics

Chromatographic separation was carried out on an ACQUITY Premier CSH C18 column (2.1 mm x 100 mm, 1.7 μ m, Waters, USA) at a flow rate of 0.3 mL/min. The mobile phase consisted of H₂O:ACN (40:60; mobile phase A) and isopropanol:ACN (9:1; mobile phase B), which were modified with a total buffer concentration of 10 mM ammonium acetate and 0.1% acetic acid. The following gradient (23 min total run time, including re-equilibration) was applied (min/%B): 0/15, 2.5/30, 3.2/48, 15/82, 17.5/99, 19.5/99, 20/15, 23/15. Column temperature was maintained at 65 °C, the autosampler was set to 4 °C and sample injection volume was 2 μ L. Analytes were recorded via a full scan with a mass-resolving power of 120,000 over a mass range from 200 – 1700 m/z (scan time: 100 ms, RF lens: 70%) in polarity-switching mode. To obtain MS/MS fragment spectra, data-dependant acquisition was carried out (resolving power: 15,000; scan time: 54 ms; stepped collision energies [%]: 25/35/50; cycle time: 600 ms). Ion source parameters were set to the following values: spray voltage: 3,250 V/ 3,000 V, sheath gas: 45 psi, auxiliary gas: 15 psi, sweep gas: 0 psi, ion transfer tube temperature: 300 °C, vaporizer temperature: 275 °C.

Untargeted Metabolomics (positive ionisation mode)

Chromatographic separation was carried out on an Atlantis Premier BEH Z-HILIC column (2.1 mm x 100 mm, 1.7 μ m; Waters, USA) at a flow rate of 0.25 mL/min. The mobile phase consisted of H₂O:ACN (9:1; mobile phase A) and ACN:H₂O (9:1; mobile phase B), which were modified with a total buffer concentration of 10 mM ammonium formate. The aqueous portion of each mobile phase was pH-adjusted to pH 3.0 via addition of FA. The following gradient (20 min total run time, including re-equilibration) was applied (time [min]/%B): 0/95, 2/95, 14.5/60, 16/60, 16.5/95, 20/95. Column temperature was maintained at 40 °C, the autosampler was set to 4 °C and sample injection volume was 4 μ L. Analytes were recorded via a full scan with a mass resolving power of 120,000 over a mass range from 60 – 900 m/z (scan time: 100 ms, RF lens: 70%). To obtain MS/MS fragment spectra, data-dependant acquisition was carried

out (resolving power: 15,000; scan time: 22 ms; stepped collision energies [%]: 30/50/70; cycle time: 900 ms). Ion source parameters were set to the following values: spray voltage: 4,100 V, sheath gas: 30 psi, auxiliary gas: 5 psi, sweep gas: 0 psi, ion transfer tube temperature: 350 °C, vaporizer temperature: 300 °C.

Statistical Analysis

Lipidomics and metabolomics measurements were performed in separate analytical sequences, with experimental samples randomised within each sequence. Pooled QC samples were prepared by mixing equal aliquots from each processed sample. Multiple QCs were injected at the beginning of the analysis in order to equilibrate the analytical system. A QC sample was analysed after every 5th experimental sample to monitor instrument performance throughout the sequence. For determination of background signals and subsequent background subtraction, an additional processed blank sample was recorded. Data was processed using MS DIAL 4.9.221218 (Tsugawa et al., 2015) and raw peak intensity data was normalised via total ion count of all detected features (Drotleff & Lämmerhofer, 2019). Level 1 feature identification was based on the MS-DIAL LipidBlast V68 library (lipidomics) and an in-house library for metabolomics (EMBL-MCF 2.0; Dekina et al., 2024) using accurate mass, isotope pattern, MS/MS fragmentation, and retention time information and a minimum matching score of 80%. To remove misannotations and to enhance confidence in lipid identification, intra-class elution patterns of lipid species were checked for consistency by relying on the expected chromatographic behaviour on reversed phase columns within homologous lipid series, considering carbon chain length and degree of saturation as the main factors.

After data curation, features with a CV >30% QC samples were excluded from the results. For univariate statistical analysis, data were log-transformed and unpaired t-tests were conducted between experimental groups of interest. To control for type I errors resulting from multiple testing, p-values were adjusted using the SGOF method (Carvajal-Rodríguez et al., 2009), and only features with an adjusted p-value < 0.05 were considered significant.

Raw data of the metabolomics and lipidomics analyses are available at the NIH Common Fund's National Metabolomics Data Repository website, the Metabolomics Workbench, under the study ID ST004261. Normalised data of the lung and muscle metabolomes and lipidomes are provided in Supplementary Tab. 4-9.

Differential and Pathway Analysis and Visualisation

Differentially abundant proteins, metabolites and lipids were defined by two different threshold cut-offs. To identify the most significant differentially abundant features in non-healthy compared to healthy harbour porpoises, only proteins, metabolites and lipids were considered, if adjusted p-value ($p_{\text{val}_{\text{adj}}} \leq 0.05$) and if logarithmic fold change ($FC_{\log 2} \geq 0.58$ for more abundant or ≤ -0.58 for less abundant).

For Gene Ontology (GO) term and pathway analysis, and for lipid subclass analysis, proteins, metabolites and lipids with an unadjusted p-value ≤ 0.05 and $FC_{\log 2} \geq 0.58$ or ≤ -0.58 were included to get an overview of the biological context between the two conditions.

If multiple detections of the same lipid were identified due to the different adducts of the detection in positive and negative mode, only lipids with the adduct “[M+H]⁺” or “[M+H]⁻” were kept. If a lipid was detected with both of these adducts, only the “[M+H]⁺”-associated lipid was kept.

The proteins were analysed for statistical overrepresentation of annotated GO-Slim terms in “Biological Processes” with the PANTHER classification system (reference: *Homo sapiens*; version 19.0, Thomas et al., 2022). Fisher’s exact test was used for FDR correction. Only GO-Slim terms with the most specific child term were selected instead of broader umbrella terms. For the overrepresentation analysis of the metabolites, MetaboAnalyst was used and compared against the RaMP database (version 6.0, Pang et al., 2024). Only metabolic reference pathway sets were used that contained at least two metabolite entries and statistically significant results were FDR-corrected. Significantly dysregulated lipids were analysed on a subclass level with LipidOne (reference: *H. sapiens*; version 2.3, Alabed et al., 2024). Statistical significances of differential lipid subclasses between the two conditions, non-healthy and healthy, were determined with a t-test and corrected for FDR.

Omics Integration

For omics integration, all individuals ($n = 12$) with corresponding proteomes, metabolomes and lipidomes of the lung and the muscle were included. Omics integration was performed with “mixOmics” R package (Rohart et al., 2017) and the vignette was followed as advised for supervised n-integration with “DIABLO” (Data Integration Analysis for Biomarker Discovery using Latent Variable Approaches for Omics Studies). Normalised features of the proteomes, metabolomes and lipidomes were included, if p-value ≤ 0.05 and not further filtered. For

multiple detections of the same lipid with different adducts, the same filtering procedure was followed as stated previously. Sparse Partial Least Square-Discriminant Analysis (sPLS-DA) was performed to classify the samples and identify the most crucial features for discrimination. Model tuning steps were set to include centroids as distance metric and the “Leave One Out” (LOO)-method with five folds and 50 repeats for cross-validation. Significant features and signatures were cut-off at a correlation coefficient of $r > 0.7$ which indicates strong correlation.

Results

Identification and sensitivity of omics on post-mortem harbour porpoise samples

Using a LC-MS/MS approach, we identified a total of 2560 proteins, 109 metabolites and 1093 lipids in the lung, and 1526 proteins, 110 metabolites and 1101 lipids in the muscle across non-healthy and healthy harbour porpoises. Of the identified features, only 33 proteins, two metabolites and 106 lipids in the lung and 21 proteins, 11 metabolites and 60 lipids in the corresponding muscles showed significant dysregulation in non-healthy compared to healthy individuals ($p_{\text{valadj}} \leq 0.05$, $FC_{\log2} \geq 0.58$ or ≤ -0.58 ; Fig. 1; Tab. 2).

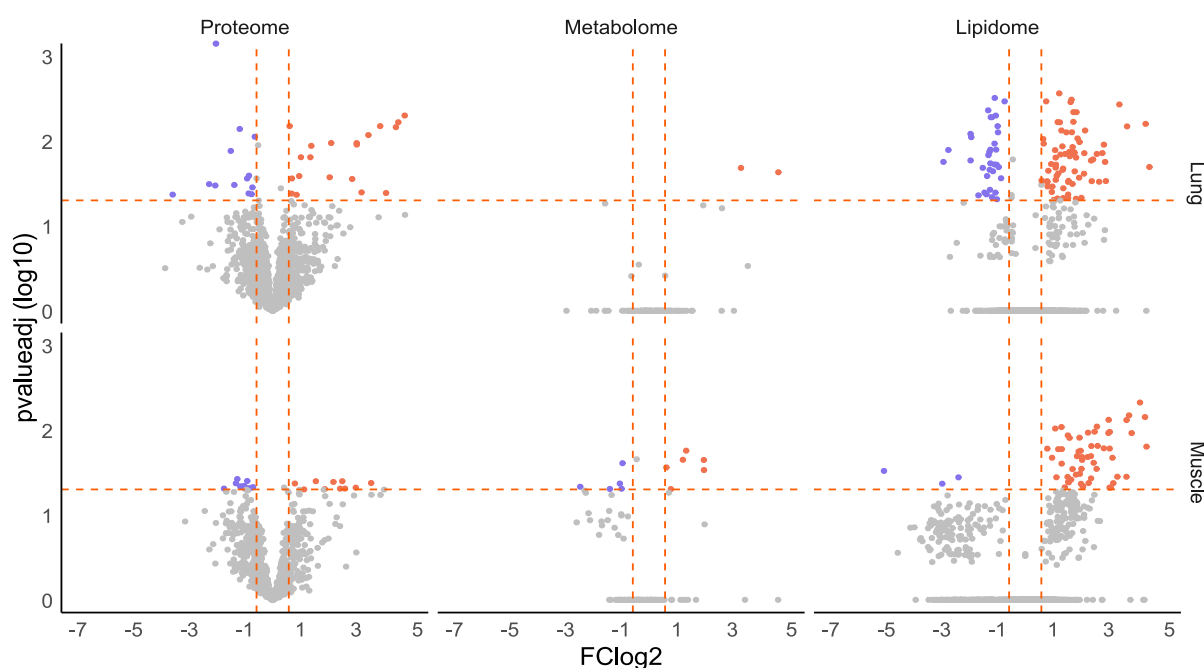


Figure 1 Differential proteins, metabolites and lipids in the lung and muscle of non-healthy, wild harbour porpoises. The x-axis indicated the log₂-transformed fold change ($FC_{\log2}$) and the y-axis the log₁₀-transformed adjusted p-value. The dashed lines mark the significance cut-offs of the adjusted p-value ($p_{\text{valadj}} \leq 0.05$) and $FC_{\log2}$ (higher abundance: $FC_{\log2} \geq 0.58$, lower abundance: $FC_{\log2} \leq -0.58$). Features are coloured according to their significant dysregulation in non-healthy harbour porpoises compared to healthy individuals: purple for less abundant features and orange for more abundant. A total of 111 not significant metabolites and lipids had $FC_{\log2}$ values outside of the here depicted range but were omitted from the graph to enhance visualisation ($p_{\text{valadj}} \geq 0.05$; lowest $FC_{\log2} = -13$, highest $FC_{\log2} = 18$).

Table 2 Overview of applied cut-offs and features included in the analyses. Cut-offs of the normalised features for the differential analysis, the pathway and GO analysis and the omics integration are defined. With the applied cut-offs, the number of proteins, metabolites and lipids in the lung and muscle that were considered for each analysis is stated. For the differential analysis and the pathway and GO analysis, the regulation in non-healthy harbour porpoises compared to healthy animals is specified.

		Dysregulated Features		Pathway and GO Analysis		Omics Integration
Cut-offs		$FC_{\log2} \geq 0.58 / \leq -0.58;$ $p_{\text{valadj}} \leq 0.05$		$FC_{\log2} \geq 0.58 / \leq -0.58;$ $p\text{-value} \leq 0.05$		$p\text{-value} \leq 0.05$
		up	down	up	down	
Lung	Proteins	20	13	114	104	2037
	Metabolites	2	0	9	2	112
	Lipids	72	34	116	50	1082
Muscle	Proteins	10	11	70	116	1135
	Metabolites	6	5	7	17	112
	Lipids	57	3	105	16	1091

MultiOmics analysis of the lung of non-healthy harbour porpoises

Proteins showed enhanced metabolic and immune processes and reduced ECM integrity

In the lung, 20 proteins were significantly enhanced, whereas 13 proteins showed significantly lower abundance in the non-healthy compared to healthy harbour porpoises (Fig. 1; Tab. 2). We identified the top up- and downregulated GO-Slim Biological Processes. Processes related to metabolism, such as purine metabolism and monocarboxylic acid metabolism, were highly elevated in non-healthy harbour porpoises (Fig. 2A, Tab. 3). Various highly abundant proteins possessed a role in oxidative stress responses, including in glutathione metabolism (GPX1, PRDX6, GPX3, ERO1A, GSTP1, SOD2, TXNRD1; Fig. 2A, Tab. 3; Supplementary Tab. 2), and innate immune responses (CTSB, DPP7, PDXK, PRDX6, C3, FLT, TCIRG1, C8A, PIN1, C4BPA, C7, PSAP, CREG1, GSTP1, PSMA4, SFTPD, MAN2B1, C6, S100A8, EPX, LAMP1, FABP5, S100A12, PNP, CTSD; Fig. 2A, Tab. 3; Supplementary Tab. 2). The most significant reduction was found for NF κ B signalling transduction, protein stability and modification processes (Fig. 2A). Other significantly reduced processes and decreased proteins were associated with extracellular matrix and transcription, such as splicing or chromatin assembly (Fig. 2A, Tab. 3).

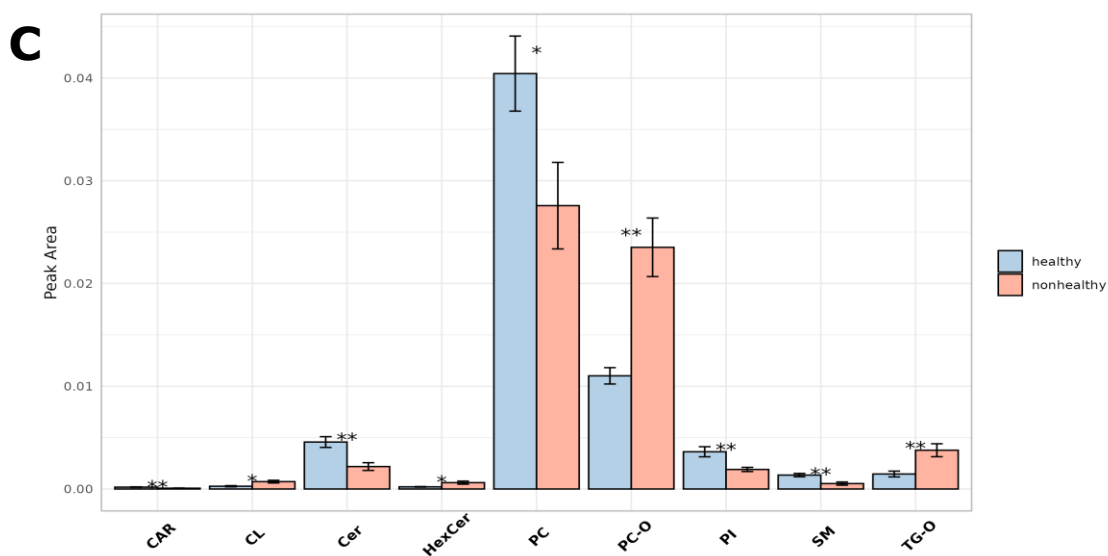
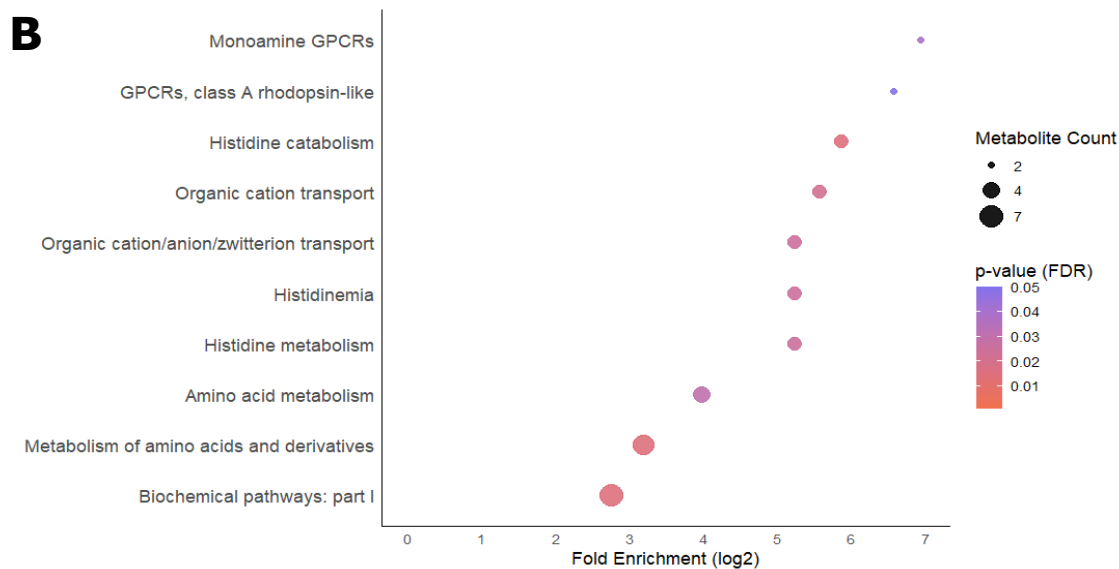
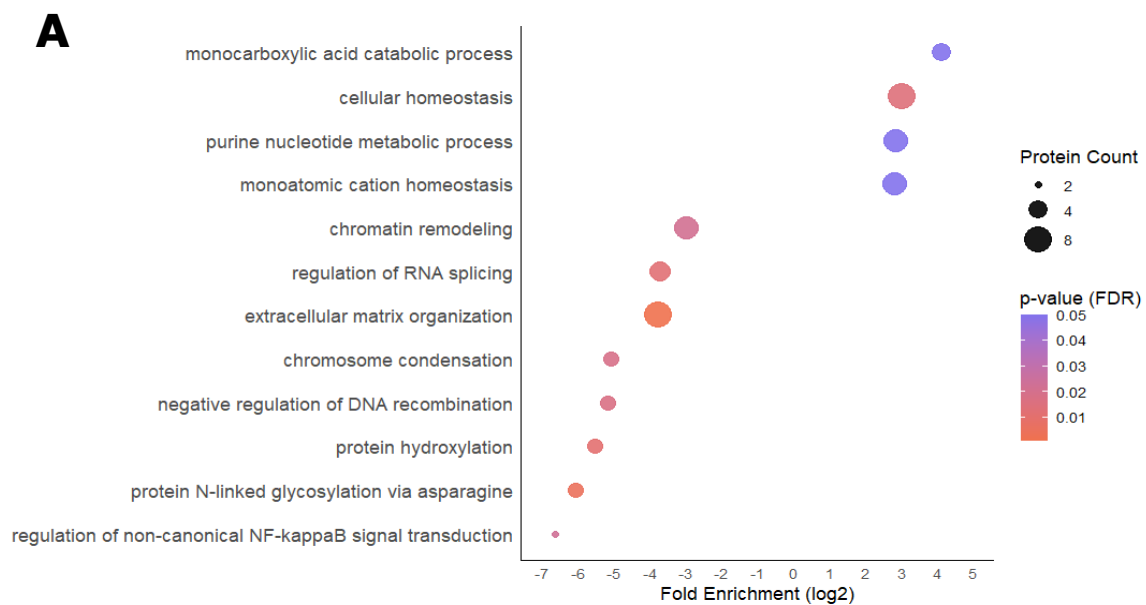


Figure 2 GO terms (proteins), pathways (metabolites) and lipid subclasses in the lung of non-healthy harbour porpoises compared to healthy individuals. Proteins, metabolites and lipids with a cut-off of $p\text{-value} \leq 0.05$, higher abundance: $FC_{\log 2} \geq 0.58$, lower abundance: $FC_{\log 2} \leq -0.58$ were considered. **A** Proteins were analysed for overrepresented GO-Slim “Biological process” terms using PANTHER against the reference dataset of the human. The term “Biochemical pathway: part I” encompasses all primarily metabolic pathways in mammals. **B** Metabolites were analysed with MetaboAnalyst for overrepresented metabolic pathways against the RaMP database. For A and B, the number of involved proteins or metabolites found differentially abundant in non-healthy harbour porpoises is depicted by increasing size of the dot. Significance of the respective pathways and processes is indicated by a colour scale. Fold enrichment was $\log_2$ -transformed. **C** Lipid subclass composition of significantly altered lipids between the conditions was analysed with LipidOne. Not significant subclasses were excluded. The peak area is a measure for lipid abundance. Significant differences between conditions were calculated with an ANOVA or t-test and are indicated by asterisks (* < 0.05 , ** < 0.01). CAR: carnitine, CL: cardiolipin, CER: ceramide, HexCer: hexosylceramide, PC: phosphatidylcholine, PC O-: ether-phosphatidylcholine, PI: phosphatidylinositol, SM: sphingomyelin, TG O-: ether-triacylglycerol.

Metabolites showed elevated histamine and histidine metabolism

In lungs of non-healthy harbour porpoises, metabolic pathways related to amino acid metabolism, such as histidine, and transport processes (e.g., monoamine GPCRs, organic cation transport) were elevated (Fig. 2B). Only serotonin and methylhistamine were significantly elevated in non-healthy harbour porpoises compared to the healthy control (Tab. 3).

Lipids showed high abundance of ether-linked lipids and reduced lipids integral to membrane and lipid raft formation

The analysis of differential lipid subclasses with LipidOne indicated a significantly altered composition for nine of 19 dysregulated subclasses between non-healthy and healthy porpoises (Fig. 2C). These included ether-linked lipid subclasses, such as ether-phosphatidylcholines (PC O-), that showed high elevation in non-healthy individuals compared to their healthy counterparts (Fig. 2C, Tab. 3). Cardiolipins along with intermediate lipids, were also found significantly elevated in non-healthy individuals (LPG 22:5, PG 22:4_22:6; Fig. 2C, Tab. 3). Significant reduction was found for lipids involved mainly in membrane integrity and fluidity (PC, Cer, SM, PI) and fatty acid metabolism (CAR, FA; Fig. 2C, Tab. 3).

Table 3 Top 10 significantly dysregulated proteins, metabolites and lipids in the lung of non-healthy harbour porpoises. Proteins, metabolites and lipids were considered highly significantly dysregulated if adjusted p-value ($p\text{-val}_{\text{adj}}$) < 0.05 and logarithmic fold change ($\text{FC}_{\log 2}$) > 0.58 for more abundant and < -0.58 for less abundant features in non-healthy porpoises in comparison to healthy porpoises. Protein IDs, names and function were inferred from the UniProt protein database for the vaquita (*Phocoena sinus*). Metabolites and lipids were identified with MS-Dial against a metabolite and lipid database. For metabolites, the identification number of the Human Metabolite database (HMDB) is given, which was used to define the function. The function of methylhistamine was not exactly determinable and was inferred from 1-methylhistamine (HMDB0000898). For the lipids, only the function of the lipid subclass of significantly dysregulated lipids could be given. To describe the function, the lipid subclass descriptions of lipotype.com were used. The statistical significance was calculated with a student's t-test for proteins and with the sequential goodness of fit test for metabolites and lipids.

	Protein ID	Protein Name	$\text{FC}_{\log 2}$	$P\text{-val}_{\text{adj}}$	Function
Protein	higher abundance				
	A0A8C9DW40	Protein dispatched homolog 1	4.75	0.005	hedgehog signalling, transporter activity
	A0A8C9CLS8	Ig-like domain-containing protein	4.52	0.006	immune system
	A0A8C9CZF6	Mitochondrial proton/calcium exchanger protein	4.44	0.007	mitochondrial structure maintenance, ion antiport mediator
	A0A8C9E9X4	Polyadenylate-binding protein 1-like 2	4.08	0.041	RNA binding
	A0A8C9CL25	Ig-like domain-containing protein	3.87	0.007	immune system
	A0A8C9C8S6	Ig-like domain-containing protein	3.44	0.009	immune system
	A0A8C9CPN9	Ig-like domain-containing protein	3.2	0.04	immune system
	A0A8C9BJ02	Matrix Gla protein	3.03	0.011	matrix protein, bone formation inhibitor
	A0A8C9C8T6	Glutathione peroxidase	3.03	0.011	oxidative stress response, selenoprotein
	A0A8C9E837	Ig-like domain-containing protein	2.86	0.028	immune system
	lower abundance				
	A0A8C9BZR1	Collagen alpha-1(XII) chain	-3.6	0.043	interacts with type I-collagen fibrils
	A0A8C9BTE7	Collagen alpha-1(XIV) chain	-2.28	0.032	forms collagen bundles, may interact with matrix or surface receptors
	A0A8C9ECV4	Fibulin-5	-2.07	0.033	elastic fibre formation and stabilisation, may be involved in vascular development and remodelling
	A0A8C9CMA7	H1.5 linker histone, cluster member	-1.51	0.013	chromatin fibre organisation
	D2CSH6	MHC class II antigen	-1.38	0.033	immune response, antigen presentation
	A0A8C9BC52	H1.3 linker histone, cluster member	-1.18	0.007	chromatin fibre organisation
	A0A8C9B9F6	Lamin B1	-0.92	0.028	nuclear assembly, nuclear membrane integrity, chromatin formation
	A0A8C9CCD0	Aspartyl/asparaginyl beta-hydroxylase	-0.86	0.041	hydroxylates asparagine or aspartic acid at EGF domains or proteins

	A0A8C9B6W2	Laminin subunit beta-1	-0.85	0.026	interacts with ECM components, membrane assembly
	A0A8C9CJH6	Nidogen-1	-0.76	0.042	basement membrane component, associates with laminin and collagen, cell-ECM interaction
Metabolite	Metabolite ID	HMDB ID	FC_{log2}	P-val_{adj}	Function
	higher abundance				
	Serotonin	HMDB0000259	4.66	0.023	neurotransmitter, behaviour modulator
	Methylhistamine	nd	3.31	0.021	histidine metabolism
Lipid	Lipid ID	Lipid Subclass	FC_{log2}	P-val_{adj}	Subclass Function
	higher abundance				
	PC O-18:2_22:6	Ether-Phosphatidylcholine	4.47	0.02	eicosanoid biosynthesis, inflammatory response
	PC O-15:1_16:1		4.33	0.006	
	PC O-22:6_22:6		3.66	0.007	
	PC O-36:8		2.87	0.018	
	PC O-15:1_14:0		2.83	0.011	
	PC O-22:6_20:4		2.77	0.014	
	PC O-14:1_16:1		2.66	0.03	
	PC O-36:9		2.58	0.014	
	LPG 22:5	Lyso-phosphatidyl-glycerol	3.38	0.004	precursor for PGs
	PG 22:4_22:6	Phosphatidylglycerol	2.9	0.03	key intermediate of cardiolipin biosynthesis, mitochondrial membrane formation, innate immune response
	lower abundance				
	Cer 18:1;O2/22:1(2OH)	Ceramide	-2.94	0.018	lipid raft formation, cell signalling, differentiation, proliferation and homeostasis
	Cer 18:2;O2/24:1(2OH)		-2.77	0.013	
	Cer 18:1;O2/24:1;O		-1.97	0.008	
	Cer 18:1;O2/24:1(2OH)		-1.94	0.009	
	SM 24:0;O2(FA 18:1)		-1.97	0.017	
	SM 24:0;O2(FA 17:0)	Sphingomyelin	-1.53	0.021	key cell membrane function, lipid raft formation
	SM 26:0;O2(FA 18:1)		-1.47	0.04	
	CAR 24:1	Acylcarnitine	-1.4	0.042	fatty acid β -oxidation
	CAR 26:1		-1.37	0.026	
	FA 18:4	Fatty acid	-1.69	0.044	cellular energy source

MultiOmics analysis of the muscle of non-healthy harbour porpoises

Proteins showed elevated muscle structure pathways and decreased β -oxidation

Significantly elevated proteins were mainly involved in muscle function and structure (Fig. 3A; Tab. 4). Multiple PDLIM family proteins with a muscular structure function (PDLIM7, LDB3, PDLIM5) and transport-related proteins were significantly more abundant in muscles of non-healthy harbour porpoises (Tab. 4). The highest upregulation in the muscle was found for the same protein in the lung, protein dispatched homolog 1 (Tab. 3, 4). Various biological processes were significantly reduced, including metabolism, translation and mitochondrial structure (Fig. 3A). Most downregulated terms were associated with lipid metabolism, specifically β -oxidation (STOML2, FABP3, ETFDH) and including both subunits of fatty acid metabolism key mediator, trifunctional enzyme (HADHA, HADHB; Fig. 3A, Tab. 4). Further reduced processes included protein folding and translation (Fig. 3A, Tab. 4) and the most significantly reduced proteins were chaperone complex proteins that assist in correct protein assembly (CCT5, CCT2; Tab. 4).

Metabolites showed dysregulation of incomplete protein metabolism and transport processes

Muscles of non-healthy harbour porpoises were mostly enriched for histidine and histamine metabolism and selen-associated metabolic pathways (Fig. 3B, Tab. 4). Similar to the proteome, dysregulated metabolites in the muscles of non-healthy harbour porpoises showed a reduced enrichment for amino acid metabolism, transcriptional and translational processes. Most terms that showed a significant reduction were associated with transport activities (e.g., amino acid transport defects, Na^+/Cl^- -dependent neurotransmitter transporters). In concordance, instable and intermediate protein metabolism metabolites, valyl-lysine and isovaleryl carnitine, were also highly enhanced (Tab. 4). Moreover, isoleucine, leucine and cytidine, metabolites related to fatty acid metabolism, exhibited lower abundance (Tab. 4).

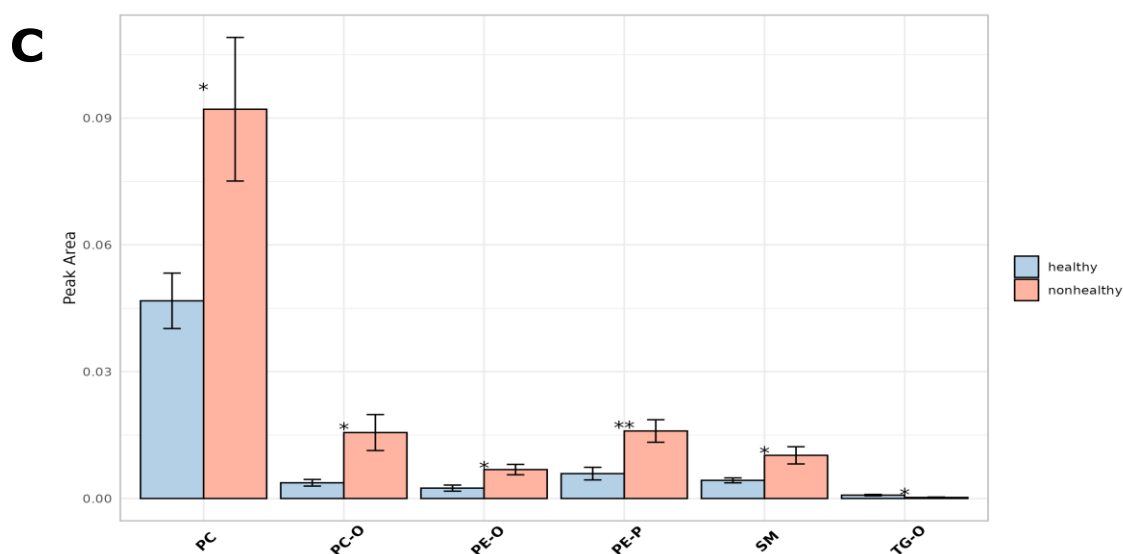
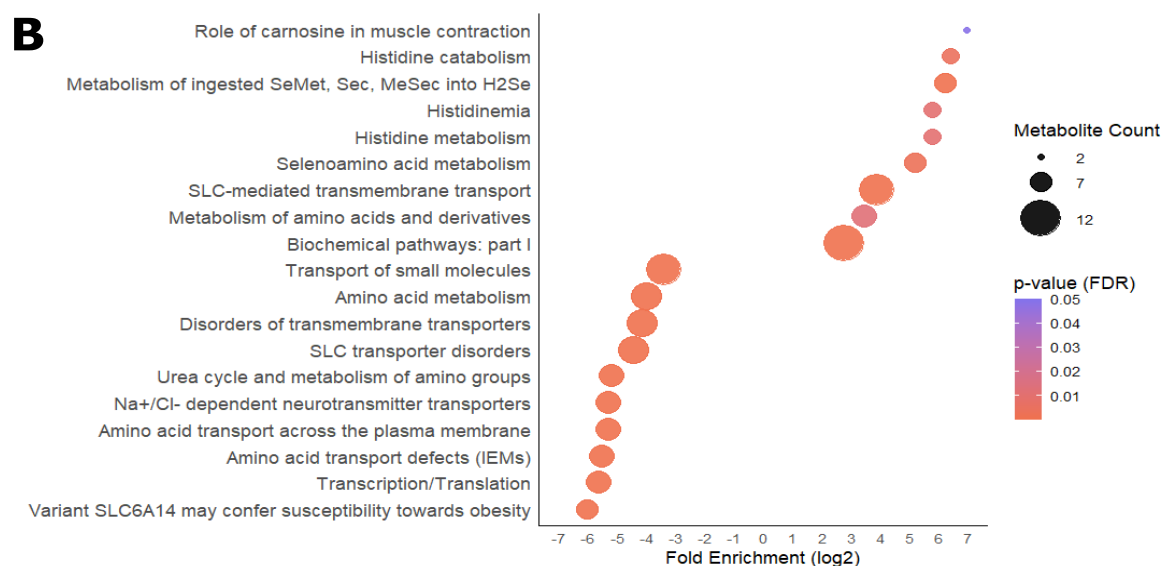


Figure 3 GO terms (proteins), pathways (metabolites) and lipid subclasses in the muscle of non-healthy harbour porpoises compared to healthy individuals. Proteins, metabolites and lipids with a cut-off of $p\text{-value} \leq 0.05$, higher abundance: $FC_{\log 2} \geq 0.58$, lower abundance: $FC_{\log 2} \leq -0.58$ were considered. **A** Proteins were analysed for overrepresented GO-Slim “Biological process” terms using PANTHER against the reference dataset of the human. **B** Metabolites were analysed with MetaboAnalyst for overrepresented metabolic pathways against the RaMP database. For A and B, the number of involved proteins or metabolites found differentially abundant in non-healthy harbour porpoises is depicted by increasing size of the dot. Significance of the respective pathways and processes is indicated by a colour scale. Fold enrichment was $\log_2$ -transformed. **C** Lipid subclass composition of significantly altered lipids between the conditions was analysed with LipidOne. Not significant subclasses were excluded. The peak area is a measure for lipid abundance. Significant differences between conditions were calculated with an ANOVA or t-test and are indicated by asterisks (*: < 0.05 , **: < 0.01). PC: phosphatidylcholine, PC O-: ether-phosphatidylcholine, PE O-: oxidised ether-phosphatidylethanolamine, PE P-: ether-phosphatidylethanolamine, SM: sphingomyelin, TG O-: ether-triacylglycerol.

Lipids showed phospholipids and sphingomyelins as highly abundant

Significantly differentially abundant lipids were classified into a total of 20 identified lipid subclasses. Of these, six showed a significant difference between conditions (Fig. 3C). Except for ether-triacylglycerols (TC O-), all lipid subclasses were significantly more abundant in non-healthy harbour porpoises. These included main membrane lipids, such as phosphatidylcholines and sphingomyelins. Plasmalogens (PC O-, PE O-, PE P-) showed a high increase in the muscles of non-healthy individuals compared to healthy porpoises (Fig. 3C, Tab. 4).

Table 4 Top 10 significantly dysregulated proteins, metabolites and lipids in the muscle of non-healthy harbour porpoises. Proteins, metabolites and lipids were considered highly significantly dysregulated if adjusted $p\text{-value}$ ($p\text{-val}_{\text{adj}}$) < 0.05 and logarithmic fold change ($FC_{\log 2}$) > 0.58 for more abundant and < -0.58 for less abundant features in non-healthy porpoises in comparison to healthy porpoises. Protein IDs, names and function were inferred from the UniProt protein database for the vaquita (*Phocoena sinus*). Metabolites and lipids were identified with MS-Dial against a metabolite and lipid database. For metabolites, the identification number of the Human Metabolite database /HMDB is given, which was used to define the function. The function of methylhistamine was not exactly determinable and was inferred from 1-methylhistamine (HMDB0000898). For the lipids, only the function of the lipid subclass of significantly dysregulated lipids could be given. To describe the function, the lipid subclass descriptions of lipotype.com were used. The statistical significance was calculated with a student's t-test for proteins and with the sequential goodness of fit test for metabolites and lipids.

	Protein ID	Protein Name	$FC_{\log 2}$	$P\text{-val}_{\text{adj}}$	Function
Protein	higher abundance				
	A0A8C9DW40	Protein dispatched homolog 1	3.54	0.042	hedgehog signalling, transporter activity
	A0A8C9C1S1	PDZ and LIM domain 7	2.99	0.048	muscle structure development
	A0A8C9E4R0	Ferritin	2.6	0.049	iron storage and transport, ferroxidase activity
	A0A8C9DZZ6	Telethonin	2.5	0.04	muscle assembly
	A0A8C9BQN0	Ferritin	2.43	0.049	iron storage and transport, ferroxidase activity
	A0A8C9B518	Synaptopodin-2	2.19	0.041	actin bundle formation
	A0A8C9BSZ6	LIM domain binding 3	1.88	0.05	

A0A8C9BSD6	LIM domain binding 3	1.55	0.04	muscle structure development, actin cytoskeleton organisation
A0A8C9E168	VPS35 endosomal protein-sorting factor-like	1.13	0.05	endocytic cargo recycling,
A0A8C9AU9	PDZ and LIM domain 5	0.8	0.043	muscle structure development, actin cytoskeleton organisation
lower abundance				
A0A8C9DZI4	Leucine-rich PPR motif-containing protein, mitochondrial	-1.75	0.049	mitochondrial RNA metabolism, associated with ubiquitin-dependent autophagy
A0A8C9CQB2	Stomatin-like protein 2, mitochondrial	-1.32	0.042	mitochondrial activity and biogenesis regulation, cardiolipin biosynthesis, involved in various cell stress responses
A0A8C9BEN4	DnaJ homolog subfamily C member 11	-1.27	0.038	mitochondrial membrane organisation
A0A8C9BL23	Trifunctional enzyme subunit alpha, mitochondrial	-1.16	0.046	long-chain fatty acid β -oxidation, cardiolipin remodelling
A0A8C9BL60	Trifunctional enzyme subunit beta, mitochondrial	-1.1	0.046	long-chain fatty acid β -oxidation, lncRNA binding
A0A8C9B732	T-complex protein 1 subunit epsilon	-1.02	0.046	chaperone complex component, protein folding, telomere maintenance
A0A8C9BKU5	Fatty acid binding protein 3	-1	0.049	long-chain fatty acid and lipid transport
A0A8C9EBA4	Nucleoside diphosphate kinase	-0.92	0.04	nucleoside diphosphate synthesis
A0A8C9B3W6	Electron transfer flavoprotein-ubiquinone oxidoreductase	-0.88	0.049	fatty acid β -oxidation, ubiquinone binding
A0A8C9CDM9	T-complex protein 1 subunit beta	-0.78	0.048	chaperone complex component, protein folding, telomere maintenance

Metabolite	Metabolite ID	HMDB ID	FC _{log2}	P-val _{adj}	Function
	higher abundance				
	Methylhistamine	nd	1.98	0.03	histidine metabolism
	Histamine	HMDB0000870	1.98	0.022	vasodilator, neurotransmitter
	1-Methylnicotinamide	HMDB0000699	1.34	0.017	essential primary metabolite, energy source
	Isovalerylcarnitine	HMDB0000688	1.22	0.022	metabolic by-product of isovaleric acidemia
	Valyl-Lysine	HMDB0029132	0.79	0.05	incomplete by-product of protein metabolism
	Nicotinamide	HMDB0001406	0.63	0.028	vitamin B3 form, preform of 1-Methylnicotinamide
	lower abundance				
	Cytidine	HMDB0000089	-2.47	0.047	nucleoside, involved in PC and PE biosynthesis
	Proline	HMDB0000162	-1.41	0.049	protein biosynthesis, collagen component
	Isoleucine	HMDB0000172	-1.05	0.043	branched chain amino acid, ketogenic and glucogenic energy metabolism

Lipid	Leucine	HMDB0000687	-0.98	0.049	branched chain amino acid, ketogenic energy metabolism, muscle protein biosynthesis, stress
	Pyridoxine	HMDB0000239	-0.95	0.025	vitamin B6 form, blood cell production, macromolecule catabolism
	Lipid ID	Lipid Subclass	FC_{log2}	P-val_{adj}	Subclass Function
	higher abundance				
	PC O-22:6_22:6	Ether-Phosphatidylcholine	5.23	0.009	eicosanoid biosynthesis, inflammatory response
	PC O-18:5_22:6		4.13	0.005	
	PC O-22:6_20:4		3.83	0.011	
	PC O-37:7		3.73	0.007	
	PC O-18:2_22:6		3.64	0.035	
	LPC O-18:1	Ether-Lyso-phosphatidylcholine	4.36	0.016	precursors of PCs, inflammatory response
	LPC O-16:1		3.31	0.035	
	LPC O-18:5		3.17	0.045	
	PG 22:6_22:6	Phosphatidylglycerol	4.31	0.007	key intermediate of cardiolipin biosynthesis, mitochondrial membrane formation, innate immune response
	BMP 22:6_22:6	Bis-(monoacylglycero)-phosphate	3.64	0.008	membrane docking and digestion, vesicle function
	lower abundance				
	TG 22:1_22:1_22:5	Triacylglycerol	-5.08	0.03	source for structural and bioactive lipids and energy
	PC O-36:0	Ether-Phosphatidylcholine	-2.99	0.043	eicosanoid biosynthesis, inflammatory response
	TG O-18:0_16:0_18:1	Ether-Triacylglycerol	-2.4	0.036	bioactive, structural lipids, energy source

Dysregulated cross-tissue features in the lung and muscle of non-healthy harbour porpoises

We analysed features for similar dysregulation in both, the lung and the muscle ($p\text{-value} \leq 0.05$; $FC_{\log2} \geq 0.58$ and ≤ -0.58). Figure 4 depicts Venn diagrams that illustrate cross-tissue dysregulated features for each omics layer. Comparing dysregulated proteins from the lung with those of the muscle, 13 proteins showed upregulation in both and five were downregulated. Interestingly, five of the upregulated proteins across both tissues play an important role in antioxidant defence and detoxification (PRDX6, GLO1, GSTP1, ferritin; Fig. 4A). Moreover, five immunoglobulins showed an increase in abundance in both tissues. Notably, four of these immunoglobulins were also among the most elevated proteins in the lung, while both ferritins

were under the top 10 upregulated proteins in the muscle (Tab. 3, 4). CTSD is involved in protein breakdown, HINT1 is a hydrolase associated with purine nucleotides. The five concordantly decreased proteins are involved in nuclear processes and cell cycle (SFPQ, CENPV) and membrane and ECM stability (DNAJC11, HSPG2, SERPINH1).

Proteins that exhibited inconsistent cross-tissue regulation and were upregulated in the lung but downregulated in the muscle were involved in metabolite detoxification (ECHDC1), structure maintenance (LETM1, DSTN) and β -oxidation (ECI2).

Histamine, methylhistamine and isovalerylcarnitine were identified to be highly elevated metabolites in the lung and in the muscle of non-healthy harbour porpoises and were also among the significantly upregulated metabolites in the muscle (Fig. 4B; Tab. 4).

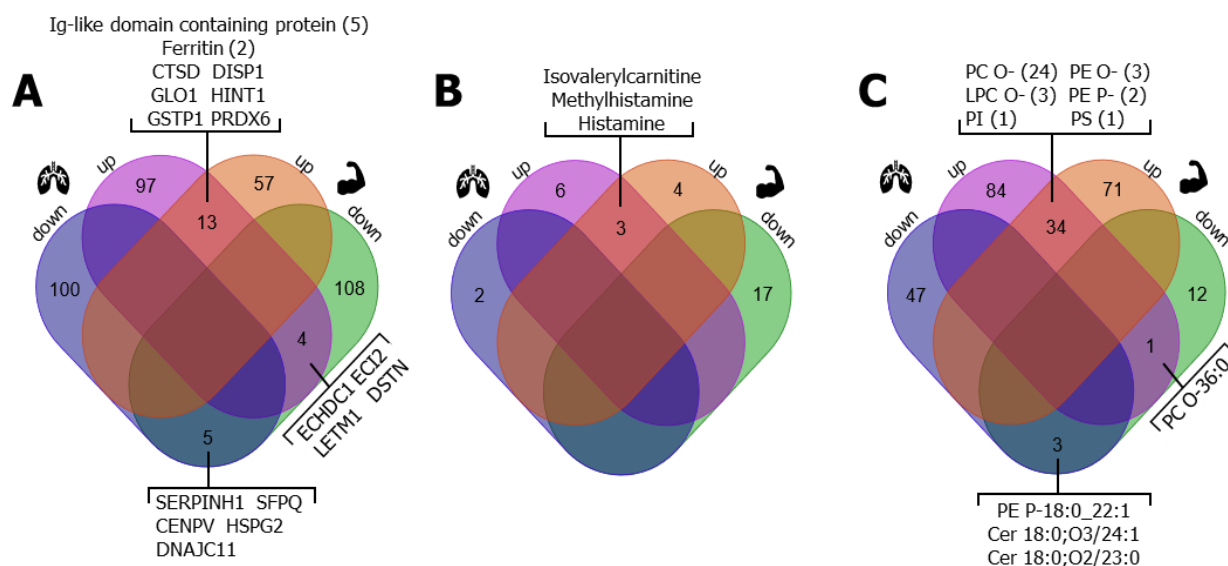


Figure 4. Venn diagrams of similarly dysregulated proteins, metabolites and lipids between the lung and muscle. Features were considered if p -value < 0.05 and $FC_{\log_2} > 0.58$ (more abundant) or < -0.58 (less abundant) in the tissues of non-healthy compared to healthy harbour porpoises. On the left side of the Venn diagrams, the upregulated (purple) and downregulated (blue) features are shown, while on the right side, upregulated (orange) and downregulated (green) features of the muscle are highlighted. **A** depicts similarly dysregulated proteins in the lung and muscle. Of the 22 identified proteins, 13 were upregulated in both tissues and five proteins were found to be decreased in the lung and muscle. Four proteins showed dissimilar regulation and were upregulated in the lung, but downregulated in the muscle. The numbers in brackets behind ferritin and Ig-like domain containing protein indicate the number of unique proteins that were identified with this name but could not be further specified. **B** shows overlapping metabolites in both tissues. Only three cross-tissue metabolites were identified, all of which were upregulated in both tissues. **C** shows the Venn diagram for the lipids of the lung and muscle. Due to the large number, cross-tissue upregulated lipids were summarised into lipid subclasses. Of a total of 38 identified lipids, 34 were elevated in both tissues and encompassed six subclasses. Three lipids were found decreased in both tissues and one lipid exhibited dissimilar regulation. The numbers in brackets represent the number of identified lipids of this subclass, respectively. Cer: ceramide, PC O-: ether-phosphatidylcholine, PE O-: oxidised ether-phosphatidylethanolamine, PE P-: ether-phosphatidylethanolamine, PI: phosphatidylinositol, PS: phosphatidylserine.

A total of 38 lipids were found similarly dysregulated in both tissues in non-healthy harbour porpoises, of which the majority was highly elevated (Fig. 4C). The 34 elevated lipids were summarised to subclasses, which were mainly ether-linked lipid classes. Most identified lipids belonged to ether-phosphatidylcholines (24 lipids) and to a lesser extent to other plasmalogens (5 lipids) or subclasses (LPC O-, PI, PS: phosphatidylserine). Two ceramides and one plasmalogen were less abundant in both tissues, while one ether-phosphatidylcholine exhibited nonconcurrent regulation across both tissues. plasmalogen were less abundant in both tissues, while one ether-phosphatidylcholine exhibited nonconcurrent regulation across both tissues.

Integrated MultiOmics analysis and signature in the lung and muscle

By integrating normalised abundance data of all omics layers for each tissue with mixOmics “DIABLO”, we defined key features for a signature that best explains the molecular differentiation between non-healthy and healthy harbour porpoises. Since our sample size consisted of six individuals in each group, we restricted the number of contributing features to six in the top two components for each omics set. The discrimination between non-healthy harbour porpoises and healthy harbour porpoises was assessed with a sPLS-DA (Fig. 5A, 6A). Clustering of the conditions was more discrete in the lung than in the muscle, especially for the metabolite and lipid blocks (Fig. 5A, 6A). One non-healthy harbour porpoise, Pph_nh6, and one healthy harbour porpoise, Pph_h3, clustered closely in the lung analysis. Similarly, for the metabolites and lipids in the muscle, two non-healthy (Pph_nh3, Pph_nh6) and two healthy (Pph_h3, Pph_h6) samples showed slight clustering. Next, we assessed the correlation between the omics layers (Fig. 5B, 6B). The correlation between the omics sets of the lung was high, with correlation coefficients around 0.9 indicating an appropriate design matrix that distinguishes between the health conditions (Fig. 5B).

Similar to the results of the sPLS-DA, the correlation of the muscle was lower compared to the lung, but still had high correlation coefficients of > 0.8 (Fig. 6B). The abundance profiles for all individuals further illustrate the separation of the conditions based on the five most contributing proteins, metabolites and lipids on the first component (Fig. 5C, 6C; Tab. 5). While there was a slight inter-individual variation retained for various features, mostly for metabolites including serotonin, isovalerylcarnitine and taurine in the lung, and proteins, such as A0A8C9CLS8 and A0A8C9CL25 (both encoding for Ig-like domain-containing proteins) in the muscle, the health conditions exhibited clear clustering (Fig. 5C, 6C). Again, only two samples, Pph_nh6 and Pph_h3, clustered closely together for both tissues (Fig. 5C, 6C). Lastly, we analysed the connection between the six most contributing features across the omics layers

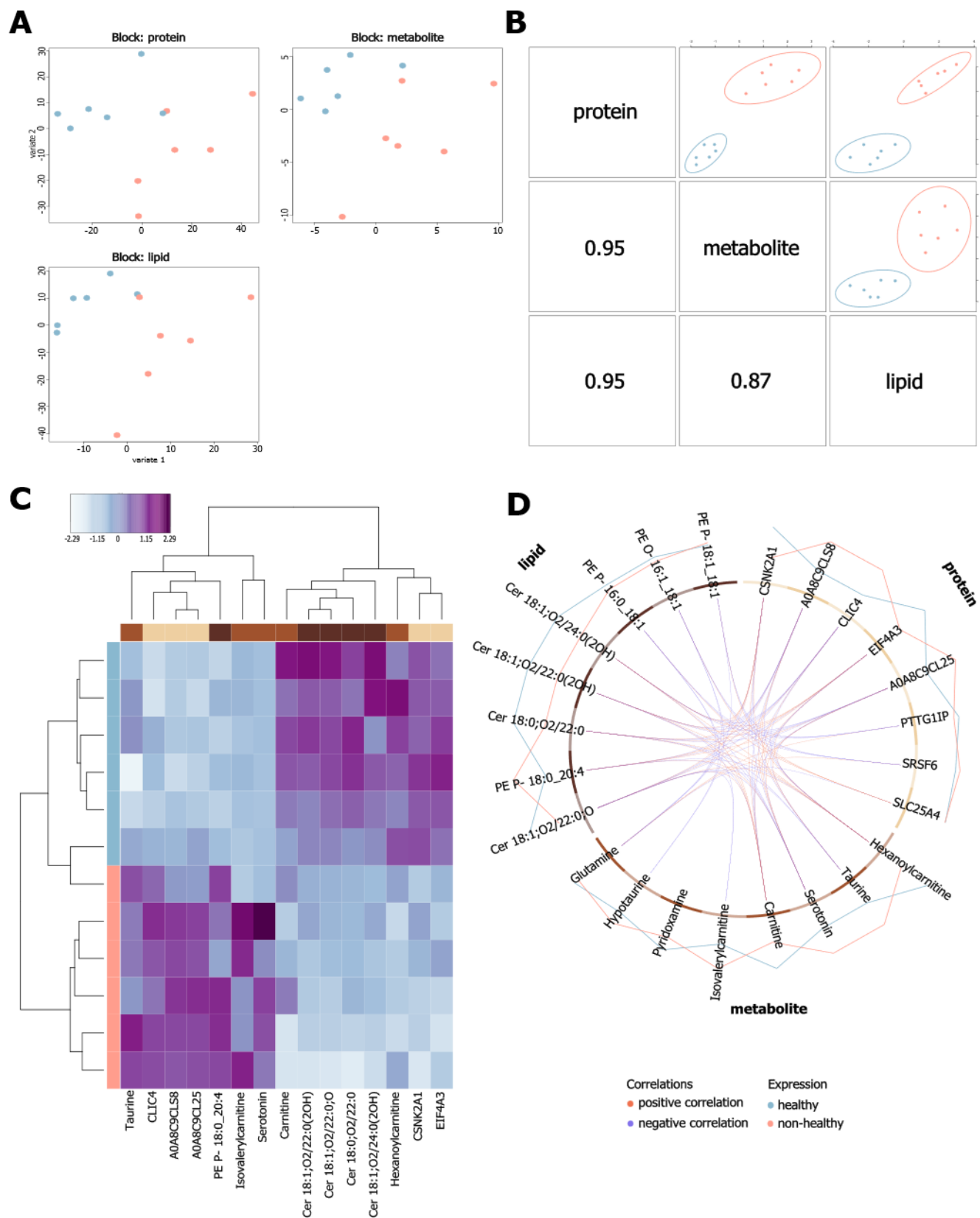


Figure 5 Integrative MultiOmics of the lung using DIABLO. Across all graphics, apricot represents non-healthy individuals, light blue depicts healthy animals. **A** shows the sPLS-DA plot of each omics layer used for omics integration, projected on both components (variant 1, variant 2). The sPLS-DA illustrates the discrimination calculated on the most contributing features between the health conditions. One non-healthy and one healthy animal share similar abundance profiles for the proteins and lipids. **B** depicts the correlations between abundance matrices of the different omics for component 1 based on the most contributing features. The plots illustrate the maximised discrimination power to distinguish between health conditions. The left-bound numbers represent the correlation coefficients of the respective pair of omics set (protein-metabolite: 0.95; metabolite-lipid: 0.87; protein-lipid: 0.95). A correlation coefficient over 0.8 indicates high correlation between omics sets. **C** Heatmap of the most discriminative features in the lung of non-healthy and healthy harbour porpoises. Individual samples are coloured vertically on the left and names of the contributing features are given at the bottom. Features types are colour-coded: proteins are beige, metabolites are brown and lipids are dark brown. Differential abundance of the features is also indicated by a colour gradient from white (low) to purple (high). **D** shows a circos plot of the correlations of the six features contributing the most to the discrimination between health conditions. Only correlations with a greater correlation coefficient of 0.7 are shown. The colour blocks each represent a different omics set (proteins: beige, metabolites: brown, lipids: dark brown). The inner lines visualise the correlations between the features, with dark orange purple indicating positive and indicating negative correlations. The outer encompassing lines represent the abundance levels of the features in the lungs of non-healthy (orange line) and healthy (blue line) harbour porpoises.

(Fig. 5D, 6D, Tab. 5, Supplementary Tab. 10). For this, we set a correlation cut-off at $r > 0.7$. Proteins, metabolites and lipids showed strong, both positive and negative, interconnected links in the lung and muscle (Fig. 5D, 6D). In the lung, A0A8C9CL25 (encoding an Ig-like domain-containing protein), taurine and two ceramides (Cer 18:1;O2/22:0;O, Cer 18:0;O2/22:0) were mostly associated with negative correlations, while SLC25A4 was only associated with positive correlations to TG O-16:0_16:0_20:4 and O-phosphoethanolamine (Fig. 5D, Tab. 5). In the muscle, anserine showed no significant correlation with any of the most contributing features. A high, positive correlation was identified for SPEG and BMP 18:1_22:6, which were both negatively correlated with pipecolic acid (Tab. 5). Notably, many metabolites (e.g., pipecolic acid, leucine, isoleucine, proline) were only negatively correlated with other contributing features (Fig. 6D).

Table 5 Correlation signature of the lung and muscle in non-healthy harbour porpoises.

Correlations between the features across the omics data were identified with mixOmics DIABLO tool which can be utilised to describe a specific marker fingerprint of a condition or disease. The regulation found in the respective omics data sets is given as logarithmic fold change ($FC_{\log 2}$). Positive and negative correlations of the features, as identified with DIABLO and visualised in Fig. 5D and 6D, is noted as well. Within the identified correlated signatures, one smaller cluster was identified in for each tissue. In the lung, this signature encompassed SLC25A4, O-phosphoethanolamine and TG O-16:0_16:0_20:4. The muscle signature combined positively correlated features SPEG and BMP 18:1_22:6 which were negatively correlated with pipecolic acid. Pipecolic acid was positively correlated with PE P-18:0_18:1 and PE P-17:0_22:6. For a more detailed table with correlation coefficients, see Supplementary Tab. 11. Nh: non-healthy, h: healthy

	Feature	Type	Regulation in nh vs h	Positive Correlation	Negative Correlation
LUNG	CLIC4	Protein	0.61		
	CSNK2A1	Protein	-2.04		
	EIF4A3	Protein	-0.64		
	SLC25A4	Protein	0.06	O-Phosphoethanolamine, TG O-16:0_16:0_20:4	
	A0A8C9CL25	Protein	3.87		
	A0A8C9CLS8	Protein	4.95		
	Carnitine	Metabolite	-0.37		
	Hexanoylcarnitine	Metabolite	-1.58		
	Isovalerylcarnitine	Metabolite	1.16		
	O-Phosphoethanolamine	Metabolite	0.24	SLC25A4, TG O-16:0_16:0_20:4	
	Serotonin	Metabolite	4.66		
	Taurine	Metabolite	0.58		
	Cer 18:0;O2/22:0	Lipid	-1.25		
	Cer 18:1;O2/22:0;O	Lipid	-1.33		
	Cer 18:1;O2/22:0(2OH)	Lipid	-1.24		
	Cer 18:1;O2/24:0(2OH)	Lipid	-1.10		
	PE P-18:0_20:4	Lipid	1.22		
	TG O-16:0_16:0_20:4	Lipid	0.31	SLC25A4, O-Phosphoethanolamine	
MUSCLE	DISP1	Protein	3.54		
	DNAJC11	Protein	-1.27		
	SPEG	Protein	0.12	BMP 18:1_22:6	Pipecolic acid
	TCAP	Protein	2.50		
	A0A8C9CL25	Protein	2.93		
	A0A8C9CLS8	Protein	3.88		
	1-Methylnicotinamide	Metabolite	1.34		
	Anserine	Metabolite	0.71		
	Isoleucine	Metabolite	-1.05		
	Leucine	Metabolite	-0.98		
	Pipecolic acid	Metabolite	0.08		SPEG, BMP 18:1_22:6
	Proline	Metabolite	-1.41		
	BMP 18:1_22:6	Lipid	1.01	SPEG	Pipecolic acid
	LPE 18:1	Lipid	1.08		
	PC O-37:7	Lipid	3.73		
	PE P-17:0_18:1	Lipid	2.26		
	PE P-18:0_18:1	Lipid	1.31	Pipecolic acid	
	PE P-17:0_22:6	Lipid	2.58	Pipecolic acid	

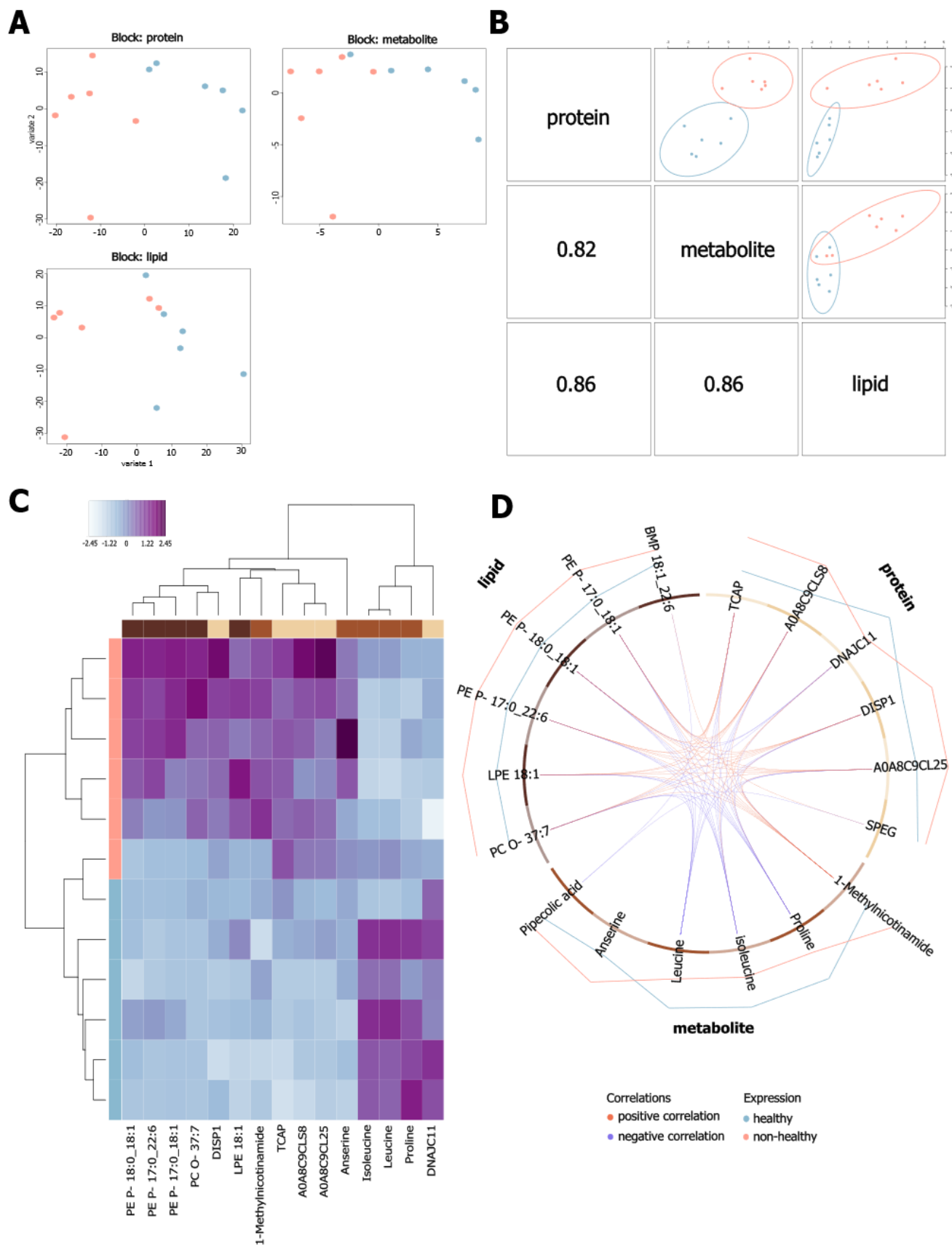


Figure 6 Integrative MultiOmics of the muscle using DIABLO. Across all graphics, orange represents non-healthy individuals, blue depicts healthy animals. **A** shows the sPLS-DA plot of each omics layer used for omics integration, projected on both components (variant 1, variant 2). Individual identification numbers of the harbour porpoises are given. The sPLS-DA illustrates the discrimination calculated on the most contributing features between the health conditions. Across the metabolites and lipids, two non-healthy and two healthy animals share similar abundance profiles. **B** depicts the correlations between abundance matrices of the different omics for component 1 based on the most contributing features. The plots illustrate the maximised discrimination power to distinguish between health conditions. The left-bound numbers represent the correlation coefficients of the respective pair of omics set, with values of > 0.8 indicating high correlation (protein-metabolite: 0.82; metabolite-lipid: 0.86; protein-lipid: 0.86). **C** Heatmap of the most discriminative features in the muscle of non-healthy and healthy harbour porpoises. Individual samples are coloured (orange: non-healthy, blue: healthy) and names of the contributing features are given. Features types are colour-coded: proteins are beige, metabolites are brown and lipids are dark brown. Differential abundance of the features is also indicated by a colour gradient from white (low) to violet (high). **D** shows a circos plot of the correlations of the six features contributing the most to the discrimination between health conditions. Only correlations with a correlation coefficient of > 0.7 are shown. The blocks each represent a different omics set (proteins: beige, metabolites: brown, lipids: dark yellow). The inner lines visualise the correlations between the features, with dark orange dark orange indicating positive and purple indicating negative correlations. The outer encompassing lines represent the abundance levels of the features in the muscle of non-healthy (orange line) and healthy (blue line) harbour porpoises.

Discussion

Necropsy material suitable for omics studies

In this study, proteins, metabolites and lipids of the lung and the muscle of free-ranging harbour porpoises, that were sampled during routine necropsies, were extracted and analysed using LC-MS/MS methods. With this omics approach, a total of 2560 proteins, 109 metabolites and 1251 lipids in the lung, and 1526 proteins, 110 metabolites and 1259 lipids in the muscle were identified. As of date, there is no comparable study of marine mammal lungs, as this is not an easily accessible and hard-to-work-with tissue. However, proteomic, metabolomic and lipidomic studies have been performed on breath exhale (blow) samples of trained bottlenose dolphins, though with less identified features (Aksenov et al., 2014; Bergfelt et al., 2018; Pasamontes et al., 2017). Many factors can influence omics analyses and yield, including extraction and detection protocols, distribution within the tissue and exact tissue sample localisation (Kershaw et al., 2018). Additionally, marine mammal reference libraries are often incomplete and still subject to ongoing studies (Mancia, 2018; Van Cise et al., 2024). This study therefore not only underlines the suitability of necropsy tissue material for omics analyses which can give timely insights into responses to environmental changes, but also adds valuable input as a reference for future studies.

MultiOmics of the lung

In the lungs of non-healthy harbour porpoises, proteins and lipids involved in anti-inflammatory and antioxidative responses were highly abundant. This included glutathione-associated proteins (GPX1, GPX3, GSTP1) among other antioxidants, such as SOD2 and PRDX6, whose important roles in protection from hypoxia and oxidative insult in marine mammals are well described (Allen & Vázquez-Medina, 2019; Cantú-Medellín et al., 2011; Tian et al., 2019, 2021a, 2021b). Diseases represent an extraordinary stress, which often also causes metabolic alterations (Kivimäki et al., 2023). Here, we found purine metabolism-associated proteins elevated (HPRT1, PRPS1, ADSL, GMPR2). Similarly, enhanced purine metabolism was observed in stressed northern elephant seals (ACTH: Deyarmin et al., 2020; fasting: Soñanez-Organis et al., 2012) and in dolphins (del Castillo Velasco-Martínez et al., 2016). A higher utilisation of the purine salvage pathway has been proposed for marine mammals, which enables them to directly recycle hypoxanthine, a reactive oxygen species-generating product of purine metabolism, for ATP production (Soñanez-Organis et al., 2012). Likewise, energetically costly translational processes were reduced in lungs of non-healthy harbour porpoises, which is often one of the first reduced processes under stress or disease to prioritise energy resources (Shah et al., 2013).

High immunoglobulin concentrations have been observed in wild animals compared to marine mammals in human care, which is presumably due to lower pathogenic impact of managed animals (Fair et al., 2017). Immunoglobulins recognise and neutralise pathogens, toxins and other foreign particles (Schroeder & Cavacini, 2010) and were also identified in a proteomic study of blow from dolphins (Bergfelt et al., 2018). In line with this, we observed high abundance of immune system proteins, including highly abundant immunoglobulins, though many could not be precisely identified due to incomplete reference data. Additionally, inflammation-alleviating lipids (plasmalogens, Brosche & Platt, 1998) and metabolites were elevated in non-healthy harbour porpoises (serotonin, methylhistamine; Herr et al., 2017; Jutel et al., 2009). With the lung as a primary interface of the organism with the environment, thus with potential environmental insults, an activation of the immune system was expected in harbour porpoises diagnosed with lung diseases. However, contrary to the anti-inflammatory T helper cell type 2 (Th2) response typically induced by pulmonary parasite infections, non-healthy harbour porpoises showed immune responses consistent with proinflammatory type 1 activation (Allen & Sutherland, 2014; Romagnani, 2006). Given that these individuals were diagnosed with severe nematode infestations and bronchopneumonia, this finding aligns with

previous studies on harbour porpoises, reporting diminished Th2 responses in severe stages (Tsang et al., 2025), possibly reflecting an immune overcompensation or a compromised immune system.

The anatomy of the cetacean lung is specialised for life under water (Piscitelli et al., 2010, 2013; Reidenberg & Laitman, 2025). Their lungs and conducting airways are enforced with thickened alveolar walls and abundant collagenous elastic fibres (Piscitelli et al., 2010, 2013). While this phenotype is indicative of pulmonary fibrosis in the human (Crouch, 1990), in whales, this is an essential diving adaptation (Kooyman & Sinnett, 1979; Piscitelli et al., 2010, 2013). Collagens are building blocks of the extracellular matrix and play a role in wound healing (Stamenkovic, 2003). Here, we found underrepresented extracellular matrix processes and ten collagens decreased in the lungs of non-healthy harbour porpoises, indicating instability of the connective tissue which is necessary to facilitate gas exchange and lung collapse. Likewise, the lungs exhibited higher abundance of cardiolipins which have been associated with lung injury and impaired surfactant function in human cell culture models of pneumonia (Ray et al., 2010; Tyurina et al., 2010). Recent similar findings in dolphin lungs further support our observation and underscore the importance of accumulated cardiolipins in impacted lungs (Porras-Gómez et al., 2025). Lung surfactant not only facilitates gas exchange, but also clears foreign compounds and pathogens from the alveolar space (Whitsett et al., 2010). Moreover, surfactant proteins have been found to be under positive selection and accelerated evolution in marine mammals (Chikina et al., 2016). While surfactant composition and concentration can be indicative of respiratory diseases, it is also altered in drowning pathology (Lorente et al., 1990) which can be applied to by-caught marine mammals. However, we could not confirm a clear distinction in surfactant protein abundance (SFTPA, SFTPB, SFTPD) between by-caught and not by-caught porpoises.

The lipid profiling showed higher abundance of ether-phosphatidylcholines and ether-triacylglycerols compared to healthy individuals. It has been stated that the cetacean lung profile is composed mainly of phosphatidylcholines, phosphatidylethanolamines, phosphatidylserines, phosphatidylinositols and sphingomyelins, regardless of their diving abilities (Arregui et al., 2020). However, lungs of non-healthy harbour porpoises displayed significantly lower abundance of phosphatidylcholines, phosphatidylinositols and sphingomyelins compared to their healthy counterparts. This may point to an altered lipid composition and inflammatory state in the pathological lung (Mehta et al., 2010), as evidenced by the proteomics data. Interestingly, one lipid compound (Cer 18:0_18:0) which was

significantly reduced in non-healthy lungs, has also been found dysregulated in the blow of a killer whale with bronchopneumonia (Harsla et al., 2024). Though this study only later diagnosed the bronchopneumonia in this animal, the similarity with our study may strengthen marker reliability to monitor health states. As surfactant is secreted by ATII cells in the lung, we cannot exclude measuring a proportion of it. Considering that marine mammals possess a surfactant composition optimised for an aquatic environment (Gutierrez et al., 2015; Miller et al., 2006; Spragg et al., 2004), and surfactant impairment has been linked with inflammatory lung diseases in humans (Griese, 1999), a combination of comparative lung tissue and surfactant analyses should be further explored to investigate potential pathological alterations.

Our results indicate a chronically inflamed lung in non-healthy harbour porpoises, along with implications of energetic imbalance and insufficient regenerative capacity. Taken together, this suggests progressive tissue loss and impairment of lung function, which may be exacerbated by a persistent cycle of sustained inflammation and inadequate repair.

MultiOmics of the muscle

The muscle is a swiftly adaptable tissue, thus possesses a high turnover rate to build and maintain tissue (Frontera & Ochala, 2015; Howard et al., 2020). This may be reflected in the high elevation for various processes and features associated with muscle structure and function in non-healthy harbour porpoises, supporting our transcriptomic results from the same animals (Dönmez et al., 2026). However, regenerative processes are energetically costly. While proteome studies of Northern elephant seal muscles suggested a strictly controlled protein turnover during fasting periods (Khudyakov et al., 2022), this may not be true for harbour porpoises. Harbour porpoises are prone to starvation due to their restricted energy reserves, temperate-to-cold habitats and high metabolic field rate, resulting in the need to constantly forage to ensure insulation and energetic supply (Koopman et al., 2002; Rojano-Doñate et al., 2024). Additionally, a diseased condition as observed here, not only manifesting in local lung inflammation, but also in a potentially chronic tissue damage and systemic inflammation, increases energetic imbalance due to the persistent activation of repair and immune pathways. Upon energy deprivation and increasing overall ATP demand, muscle tissue will be utilised as primary metabolic reservoir to meet energetic requirements (Wolfe, 2006). In accordance with this, we found pathways and metabolites pointing to protein degradation and muscle atrophy. Similar findings have been observed in the muscles of chronic obstructive pulmonary disease (COPD) patients (Jagoe & Engelen, 2003) and in the plasma of diseased dolphins (Derous et al., 2022). A potentially impaired or altered energy metabolism in muscles of non-healthy

harbour porpoises was further underlined by decreased fatty acid β -oxidation. Toothed whales feed exclusively on a high-fat diet, generate their energy primarily through lipid metabolism and have lost the ability for ketogenesis (Wolfgang et al., 2021). An impairment of their main energy pathway may force them to rely on alternative, less energetically favourable pathways such as muscle protein catabolism to provide amino acids for gluconeogenesis.

As post-mitotic tissue, muscles are prone to accumulation of oxidative injury (Frontera & Ochala, 2015; Rom & Reznick, 2016). To prevent exceeding oxidative stress, marine mammal muscles possess high baseline antioxidant concentrations (Allen & Vázquez-Medina, 2019; García-Castañeda et al., 2017; Vázquez-Medina et al., 2006). Likewise, we could identify multiple antioxidative features, including glutathione-associated proteins and selenoamino-involved metabolites. Selenoamino acids are linked to glutathione metabolism which is enhanced in marine mammals (Chung & Maines, 1981; Vázquez-Medina et al., 2006; Wilhelm Filho et al., 2002). Moreover, the high levels of anti-inflammatory and antioxidative plasmalogens in the muscle of non-healthy harbour porpoises indicate an enhanced oxidative stress state. Plasmalogens possess a unique vinyl ether-structure which enables them to bind free radicals (Brosche & Platt, 1998; Murphy, 2001) and reduced levels have been noted in inflammatory diseases (Paul et al., 2019). Although it is not possible to determine whether the increased immune and stress responses are primarily due to a possible reduction in oxygen supply caused by pathological lung lesions, chronic disease conditions, as observed in the animals studied here, will have an adverse effect on the entire body and organism.

Our findings suggest that, despite aquatic adaptations and respiratory specialisations, the persistent pulmonary immune activation contributes considerably to an elevated energy requirement with implications for the whole-body. The potential link between lung and muscle responses implies that chronic immune activation and impaired pulmonary repair mechanisms not only increase energetic costs, but may also impede oxygen availability for muscular energy generation, therefore accelerating muscle proteolysis to compensate for this systemic energy discrepancy.

Overlapping features cross tissue

Across both tissues, we could identify several, similarly regulated features in each omics set, which may be of interest in regard to downstream effects from the lung to the muscle. Again, antioxidant and immune responses were among the most common associations in highly

abundant cross-tissue regulated features (e.g., GSTP1, ferritin, immunoglobulins, histamine, methylhistamine, plasmalogens).

In both lung and muscle, the highest upregulated protein was Protein Dispatched Homolog 1 (DISP1). DISP1 is involved in hedgehog signalling, which has a role in the pathogenesis of COPD and pulmonary fibrosis, and has been proposed as COPD biomarker (Ancel et al., 2020; Cigna et al., 2012). The simultaneous upregulation of DISP1 in the pathological lung as well and corresponding muscle of non-healthy harbour porpoises may pose as valuable indicator of the systemic impact of a compromised respiratory tract.

Another interesting cross-tissue metabolite is isovalerylcarnitine, which was upregulated in both tissues of non-healthy harbour porpoises. Isovalerylcarnitine is produced during disrupted leucine catabolism, for which the significant decrease of leucine and isoleucine in the muscle of non-healthy harbour porpoises may be supporting evidence (McCalley et al., 2019; Tanaka et al., 1966). Isovalerylcarnitine can also be produced from carnitine and toxic isovaleric acid. Isovaleric acid is abundant in specialised, non-metabolised fat tissues (e.g., acoustic fats) and muscles of harbour porpoises (Koopman, 2018), whereas it is generally absent in the lung (Arregui et al., 2020; Lovern, 1934). In recent studies of acute respiratory distress syndrome and pathogen-associated pneumonia in humans, isovalerylcarnitine displayed distinctive abundance profiles between conditions and has also shown potential to be predictive of disease progression (den Hartog et al., 2024; Suber et al., 2023). As the immune responses in harbour porpoises showed similarities to those of humans, despite inhabiting different habitats, isovalerylcarnitine may also be suitable as biomarker indicative of lung diseases in harbour porpoises.

Since these dysregulated features likely circulate between tissues via the bloodstream, incorporating analyses of corresponding blood samples in future studies may deepen our understanding of multi-tissue pathophysiology and help assess their potential as biomarkers for respiratory disease in cetaceans.

Potential signature to estimate lung health and disease in marine mammals

Lastly, we aimed to identify a potential feature profile that could be utilised in a biomarker panel to monitor and estimate lung health and disease in marine mammals. We applied an integrative MultiOmics approach, including proteomics, metabolomics and lipidomics of non-healthy and healthy harbour porpoises. Integrative MultiOmics are a relatively novel method

that can provide comprehensive insights into the complex systems biology by simultaneously analysing different datasets of the same individuals (Lee et al., 2019). With mixOmics “DIABLO”, we identified a signature of a total of 18 proteins, metabolites and lipids in the lung and in the muscles that may drive the pathogenesis of lung-associated lesions in harbour porpoises. Our MultiOmics model indicated a strong interaction between SLC25A4, O-phosphoethanolamine and TG O-16:0_16:0_20:4 in the lung (Fig. 5D, Tab. 5). SLC25A4 is located in the mitochondrial membrane where it mediates ADP:ATP transport for energy generation and fuel (Cl  men  on et al., 2013), while TG O- is associated with lipid metabolism, pointing to a molecular signature in altered metabolism. In mice lungs exposed to ozone, O-phosphoethanolamine was associated with reduced hypersensitivity (Smith et al., 2023), which may validate its pathological role in the lung lesion-associated phenotype studied here. In the muscle, a signature of five features was observed within the 18 proposed features. This included a positive correlation between SPEG, a protein crucial for muscle function and myotube function (Campbell et al., 2021; Hsieh et al., 2000), and BMP 18:1_22_6, a bis-(monoacylglycero)-phosphate which are involved in vesicle trafficking (Showalter et al., 2020). The negative correlation of SPEG and BMP 18:1_22_6 with pipecolic acid, which is dysregulated in the serum of smokers and associated with oxidative stress (Dalazen et al., 2014; Shen et al., 2023), implies a compensatory response to an enhanced redox imbalance. Moreover, pipecolic acid was positively correlated with two anti-inflammatory and antioxidative plasmalogens (PE P-18:0_18:1, PE P-17:0_22:6; Brosche & Platt, 1998; Murphy, 2001; Paul et al., 2019). This interaction may indicate that elevated oxidative stress triggers a protective shift in plasmalogen metabolism, thus further supports a potentially coordinated pattern to redox stress in the muscle. Together, these features cover most of the observed dysregulated pathways we observed in the muscle of non-healthy harbour porpoises, namely elevated oxidative stress, tissue degradation and impaired regeneration. This may qualify these features as a combined panel of markers for lung-associated muscle impairments, thereby enabling the analysis of the pathological consequences on distant organs.

Integrative MultiOmics studies have been mostly applied in biomarker identification for various dispositions and diseases (Bowerman et al., 2020; Harriott et al., 2025; Ivanova et al., 2024; Konigsberg et al., 2021). Compared to these studies, our samples size is rather small and could be larger for more predictive power. Nonetheless, the identified MultiOmics signatures are a first step for biomarker identification in marine mammals and additional functional analyses will give deeper insights into the molecular mechanisms and phenotype (Lam et al., 2020).

Conclusion

Our integrative MultiOmics study on the lungs and locomotor muscles of free-ranging harbour porpoises indicates a profound and sustained immune activation in non-healthy individuals. Unlike the attenuating Th2 inflammation that is typically associated with parasitic infestation, the response reflected a proinflammatory type 1 activation, accompanied by impaired lung tissue repair, muscle atrophy and overall energetic imbalance. This molecular and metabolic dysregulation suggests reduced tissue function, potentially with direct consequences on diving ability and survival. Both lungs and muscles exhibited similarly altered abundance of several proteins, metabolites and lipids, implying systemic impacts, possibly mediated through circulation. Using mixOmics DIABLO, we identified highly correlated sets of molecules in both tissues that may serve as a lung disease-associated marker panel to support health assessments in cetaceans. Our results demonstrate that respiratory disease in harbour porpoises extends beyond the lungs and can be evident in muscles, thus affecting overall fitness and further emphasising the importance of considering systemic effects when evaluating population health. Because anthropogenic impacts, such as underwater-radiated noise, pollution and climate change, increasingly influence susceptibility to disease and resilience in cetaceans, these insights deepen the understanding of how global environmental pressures alter health and survival in marine sentinel species like the harbour porpoise.

Limitations

While decomposition has to be considered when working with necropsy tissue material, we tried to minimise potential degradation effects by following standardised best practice necropsy and sampling protocols and only considering harbour porpoises within decomposition code 2 (max. 24 h post-death). As omics are an emerging field in marine mammal science, few comparable studies are published to compare the impact of decomposition and handling on detection. One study on surfactant found little lipid composition change of post-mortem sampled marine mammals for up to two days (Gutierrez et al., 2015). We recommend that future studies should implement time-comparative experiments, where possible, to support recommendations for best practices in marine mammal omics which will certainly continue to grow. However, we were able to identify proteins, metabolites and lipids from two tissues of deceased, free-ranging harbour porpoises and compare two physiologically distinct states. We analysed the altered regulation of metabolic, stress and immune responses and provide valuable insights into the systemic implications of respiratory diseases in harbour porpoises.

Since some individuals were by-caught and non-healthy animals often suffered from several diseases, we cannot exclusively attribute the immune mobilisation in the lung and oxidative stress and metabolic response of the muscle solely to the observed lung lesions, as various diseases have an effect on multiple organs (Celli et al., 2018; Nauffal et al., 2024; Reynolds, 2002). Additionally, this study focuses on free-ranging animals whose life history (last food intake, short-term stress, recent disease recovery, and other factors) is not known but can influence molecular responses. Still, inflammation is a hallmark reaction across many pathological conditions that impacts signalling responses like interleukin or HIF1 signalling, alters metabolic pathways such as shifting to higher utilisation of anaerobic metabolism, and influences common mechanisms, e.g., tissue remodelling.

Lastly, our relatively small sample size may have an effect on the results, especially if interindividual variability plays a role. Future studies should aim for larger sample sizes for more coherent results, although this is a limiting factor in marine mammal science because these animals are elusive, difficult to sample, and often strictly protected.

Data Availability Statement

The data sets of this study can be found in the article's Supplementary Material and in online repositories. The names of the repository and accession numbers are listed in the Supplementary Material (Supplementary Tab. 1).

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CRedit Authorship Contribution Statement

EMD: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Project Administration, Validation, Visualization, Writing – Original Draft Preparation,

Writing – Review & Editing; BS: Data Curation, Formal Analysis, Investigation, Project Administration, Validation, Writing – Review & Editing; BD: Data Curation, Formal Analysis, Investigation, Project Administration, Validation, Writing – Review & Editing; PN: Data Curation, Formal Analysis, Investigation, Writing – Review & Editing; DD: Visualization, Writing – Review & Editing; AF: Conceptualization, Funding Acquisition, Supervision, Writing – Review & Editing; US: Conceptualization, Data Curation, Funding Acquisition, Resources, Supervision, Writing – Review & Editing

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Ethics Statement

Ethical review and approval were not required for the study since the investigated animals were collected once they were dead by stranding or bycaught within the German stranding network. The German stranding network conducts work, such as collecting and holding carcasses and samples from European protected species, following appropriate licenses from the relevant authorities.

8 General Discussion

8.1 An overview of the key findings

The harbour porpoises of the North and Baltic Seas experience a myriad of environmental and anthropogenic stressors that can negatively affect their health condition. Among the most affected organs are the lungs and respiratory tract, which display severe pathological lesions that are in part caused by nematode infestations. Increased burden of lung nematodes may impair tissue function and promote other comorbidities that extend beyond the pulmonary system. The skeletal muscles are essential for harbour porpoises. A shortage of oxygen supply from the lung may affect their diving ability, thus reduce foraging success and ultimately, competitiveness and resilience.

In this dissertation project, high-throughput data technologies were applied to investigate whether harbour porpoises possess molecular mechanisms to tolerate pathological lesions and parasitic insults to the respiratory organs and can compensate indirect whole-body effects on the skeletal muscles.

In **Chapter I**, transcriptomic profiling and qRT-PCR of selected candidate genes were performed to analyse whether the skeletal muscles of non-healthy harbour porpoises experience hypoxic conditions due to impaired oxygen uptake and supply of the pathological lungs. No classical hypoxia-associated response could be detected, but muscles of non-healthy harbour porpoises displayed enhanced antioxidant expression, hinting at exceeding oxidative stress. The elevation of lipid metabolic processes and rate-limiting glycolytic transcripts in the muscles of non-healthy harbour porpoises point to difficulties to manage the energetic demand with existing energy storages. Transcripts involved in tissue regeneration and selective proteasomal degradation showed high expression profiles and could be indicative of a tightly controlled switch between these processes, aiding in maintaining tissue function despite high turnover rates. A molecular, pathological effect from the respiratory organs to distant organs, such as the skeletal muscles, has not been described in harbour porpoises before. Thus, this study gave first insights into the molecular whole-body responses in harbour porpoises suffering from declining lung health.

Chapter II focuses on the pathophysiological molecular response in the lungs and whole-body effects on the muscles in non-healthy harbour porpoises which were analysed through

comparative transcriptomics, qRT-PCT and a generated *de novo* assembly. Upon pathological and parasitic insult, the lungs exhibited an immune system activation similar to the one observed in humans suffering from lung diseases. Therefore, the molecular response appears to be conserved in mammals, regardless of their inhabited environment. Furthermore, the observed gene expression patterns in the lungs of non-healthy harbour porpoises were suggestive of prolonged immune system activation which may negatively affect host defences. These findings support previous studies that observed compromised immune responses in harbour porpoises with severe infestations, as compared to beneficial Th2 immune activation in milder cases. Several transcripts showed similar dysregulation in the lungs and muscles, indicating systemic effects that should be considered in future studies. Lastly, a *de novo* transcriptome assembly allowed the identification of significantly dysregulated non-coding RNAs. These may have a regulatory effect on adaptive immune responses, which requires further validation in prospective projects.

The analysis and integration of multiple omics data sets (proteomes, metabolomes and lipidomes) of the lungs and muscles of non-healthy harbour porpoises is presented in **Chapter III**. As translation of transcripts does not necessarily reflect gene expression and bioactive molecules, such as metabolites and lipids, can affect regulatory processes, the analysis of the molecular mechanisms on several levels can give comprehensive, holistic insights. The lungs of harbour porpoises with compromised pulmonary health displayed elevated anti-inflammatory and antioxidative features. Moreover, high cardiolipin levels, which are associated with impaired lung function, and disrupted tissue integrity, as implicated by low collagen, indicate a functional impact caused by the pathological insults. Dysregulated processes of the skeletal muscles of non-healthy harbour porpoises encompassed features pointing to reduced lipid metabolism and disrupted tissue maintenance, indicative of muscle catabolism and atrophy. Hence, a systemic and functional effect of the pulmonary pathologies on peripheral end-organs such as the muscles in harbour porpoises cannot be disregarded. Such compromising downstream responses suggest that harbour porpoises may be negatively affected on a whole-organism level which ultimately could impede successful foraging and viability. Overlapping features that showed a uniform dysregulation across both tissues underline the proposed systemic impairment and peripheral circulation also observed in **chapter II**. With integrative MultiOmics, an emerging tool in marine mammal science, a promising disease-associated marker panel comprising proteins, metabolites and lipids for both

tissues could be identified. These prospective panels have to be validated experimentally and may support health estimation and monitoring efforts of free-ranging harbour porpoises.

8.2 Localised immune activation to lung nematodes: the lungs

8.2.1 Pathophysiological mechanisms in the lungs of free-ranging, non-healthy harbour porpoises

The lung serves as vital interface between the organism's first line of defence and its surrounding environment. Hence, it is equipped with a multi-layered immune response to ward off harmful, external impacts (Galeas-Pena et al., 2019; Weatherhead et al., 2020). Infection with nematode parasites is ubiquitous in marine mammals, can have serious consequences for the host's health and fitness, and put selective pressure on the host's immune responses. Studies on terrestrial animals have described that nematode infestations elicit a T helper cell type 2 (Th2) inflammatory response (Allen & Maizels, 2011; Allen & Sutherland, 2014; Maizels, 2020). Th2 inflammation generally represents an anti-inflammatory reaction, which balances parasite opsonisation and self-inflicted tissue damage due to immune activation (Fig. 4). Thus, it favours a tolerance response which may be more beneficial for overall host fitness (Allen & Sutherland, 2014). Similar findings have been observed in harbour porpoises which employ a profound Th2 inflammation in infections with mild to moderate parasite burden, but not upon heavy parasite load (Tsang et al., 2025). In line with this, the lungs of non-healthy harbour porpoises, which were characterised by more severe nematode load and diagnosed with bronchopneumonia, did not present a classic Th2 immune response. Transcriptomic expression (**Chapter II**) and proteomic profiling (**Chapter III**) of non-healthy compared to healthy porpoises were more similar to proinflammatory Th1 and Th17 immune responses and pointed to chronic inflammation (Allen & Maizels, 2011; Modolell et al., 1995; Romagnani, 2006). A suppression of beneficial Th2 responses can be caused by exceeding parasite burden or excessive, prolonged immune activation which results in a feedback loop-resembling exacerbation (Marsland et al., 2008). Since harbour porpoises feed almost constantly and are flexible in their prey species, all factors that can significantly accelerate parasite numbers and re-infection (Fischbach & Seguel, 2023), the accumulation may rapidly become overwhelming, resulting in continuous immune recruitment (Gieseck et al., 2018; Maizels, 2020). Enhanced influx of immunocytes, such as neutrophils and macrophages, has also been observed in mice and murine lung cell line models exposed to proteins of parasitic *Anisakis simplex* larvae (Cho

et al., 2010; Nieuwenhuizen et al., 2006). Such an influx leads to reduced parasite survival but also to increased inflammation and exacerbation of lung injury (Sutherland et al., 2014). Similarly, our findings suggest high recruitment of macrophages and neutrophils, as indicated by upregulation of associated chemokine and cytokine transcripts, such as interleukin 8 (IL8), a neutrophil attractant in pulmonary inflammation (**Chapter II**, Carvallo & Stevenson, 2022; Cho et al., 2010). The lungs possess various macrophages which are either recruited or stay resident and maintain tissue homeostasis amid pathological impact (Pöpperl et al., 2025). Toothed whales, such as harbour porpoises, possess a specialised macrophage which is attached to the vasculature and efficiently clears pathogenic material from the pulmonary circulation. However, these pulmonary intravascular macrophages (PIM) are also thought to induce hypersensitivity-driven inflammation, increasing vulnerability to lung injury (Anderson et al., 2021; Schneberger et al., 2012). Here, macrophagic types were not distinguished from each other, thus it is not possible to say whether and to what extent PIMs were part of the observed immune responses. Since humans do not possess PIMs, little research interest has been shown in them. Nonetheless, their presence in toothed whales should be studied further to determine whether their function is beneficial or detrimental (Kawashima et al., 2004). Cetaceans have evolved to fit their aquatic lifestyle perfectly, including adaptations of their respiratory system (Guo et al., 2024; Piscitelli et al., 2013). It is therefore reasonable to question why these animals have retained potentially harmful traits, particularly given the constant impacts their respiratory system is subjected to.

Severe lungworm infestations can promote the contraction of secondary bacterial diseases, which can invade the complete lung, since cetaceans do not possess compartmented lung lobes (Piscitelli et al., 2010, 2013; Reidenberg & Laitman, 2025). Typically, bacterial and microbial insults trigger a Th1 inflammation (Anthony et al., 2007). Thus, it is plausible that the increased Th1 activity in the lungs of non-healthy harbour porpoises is the result of co-infections, since these individuals have also been diagnosed with bacterial diseases (bronchopneumonia). Furthermore, this is supported by an increase in Th1-involved immunoglobulin gene expression and protein abundance (**Chapter II, III**). Immunoglobulins are part of the humoral immune system and mark nematodes and other invading organisms for subsequent destruction by leukocytes (Fig. 4; Chauhan et al., 2024; Kraemer et al., 2022). Immunoglobulins have also been found elevated in free-ranging compared to managed dolphins (Fair et al., 2017), underscoring the higher pathogenic exposure of free-ranging individuals and their importance in host protection.

To enable aggregation to inflamed areas, immunocytes must pass membranes and other cellular barriers which is enabled by partial degradation of the extracellular matrix (ECM) and membranes (Madri & Graesser, 2000). However, already instable tissue due to nematode migration and feeding, or exacerbating inflammation can escalate tissue degeneration and inflammation. This can further impede repair mechanisms, resulting in reduced tissue function and gas exchange capability (Ley & Zarbock, 2008). Likewise, the gene expression, protein abundance and lipid profiling results pointed towards accelerated ECM and tissue degradation, as implied by reduced collagen and altered lipid composition in non-healthy individuals (**Chapter II, III**). Healthy human lungs comprise approximately 90% of type I and III collagens (Kirk et al., 1984; Marshall et al., 1997), which are important for wound healing (Stamenkovic, 2003). In cetaceans, collagens are also essential for the specialised lung anatomy and explosive single-breath gas exchange (Kooyman & Sinnett, 1979; Piscitelli et al., 2010, 2013). Together with the significant upregulation of the gene expression and protein abundance of collagen-degrading matrix metalloproteases (MMPs; **Chapter II, III**), these findings suggest that non-healthy harbour porpoises degrade tissue, but may not be able to adequately replace it, thus drive permanent tissue loss (Hendrix & Kheradmand, 2017; LeBert et al., 2015; Pardo et al., 2016). Moreover, MMPs can be activated by proinflammatory cytokines (e.g., IL13, IL17), and *in vivo* experiments in transgenic mice have shown their role in lung inflammation and remodelling (Golestani et al., 2017), hence underline the potentially inflammation-associated upregulation in non-healthy harbour porpoises. Besides collagen, which constitutes a main proportion of the proteins in lung tissue, phosphatidylcholines account for a large portion of the lipid composition in cetacean lungs, regardless of diving profiles (Arregui et al., 2020). However, the lipidomic data of non-healthy harbour porpoises showed reduced phosphatidylcholine abundance compared to their healthy counterparts, further indicating ongoing tissue degradation (**Chapter III**). Among the lipid subclasses abundant within the lung, cardiolipins are typically absent in healthy lung tissue. However, upon injury, inflammation or disease, they can accumulate in the respiratory system (Ray et al., 2010). There, increased cardiolipin alters membrane composition and reduces elasticity, which can impair gas exchange capacity (Mehta et al., 2010; Tyurina et al., 2010). In line with these findings, lung lipid profiles of non-healthy harbour porpoises displayed significantly elevated cardiolipin abundance (**Chapter III**). Further strengthening our results, elevated cardiolipin levels have also been observed in the surfactant lipidome of managed dolphins that suffered from respiratory diseases (Porras-Gómez et al., 2025).

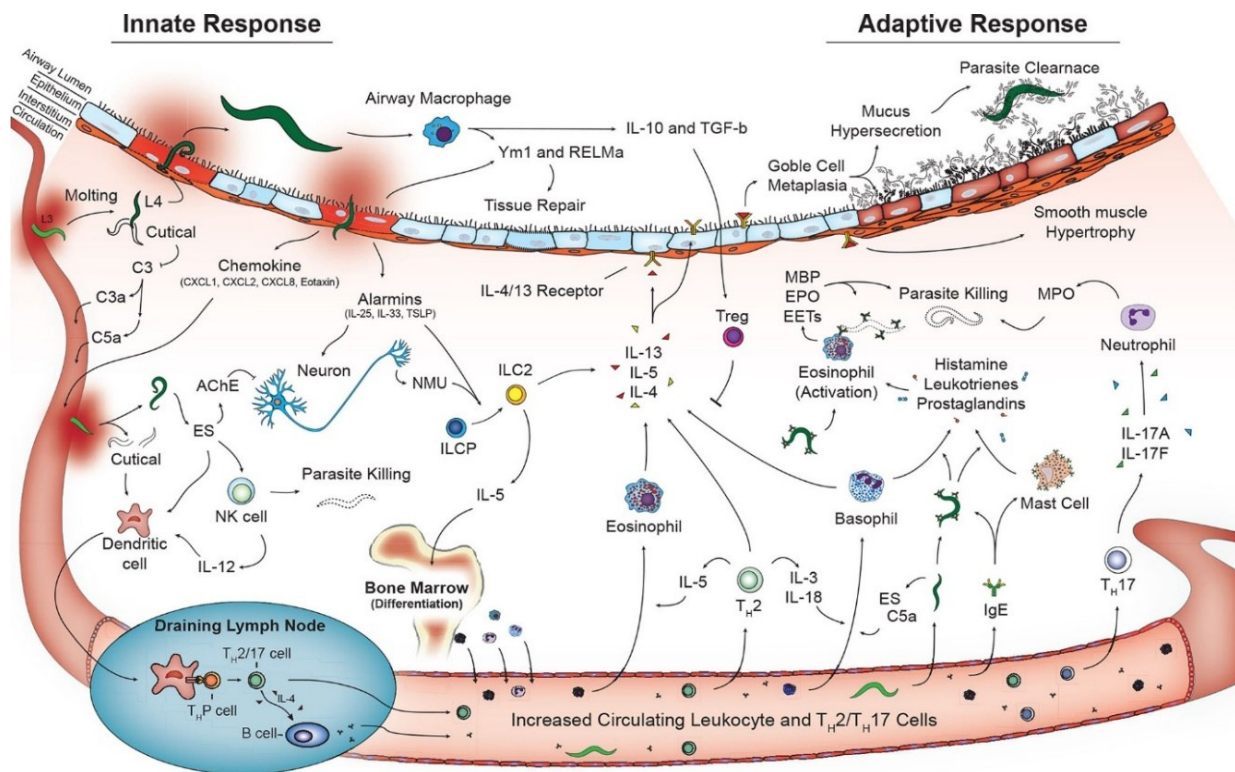


Figure 4 Respiratory host defence mechanisms upon helminth (lung nematode) infection. This graphic by Weatherhead et al. (2020, used under CC BY 4.0) visualises the mammalian host response upon infection with lung nematodes and other helminth parasites and summarises the complex and broad activation of the innate and adaptive immune system to protect the lung tissue from parasitic damage.

Similarly, surfactant abnormalities have previously been linked to inflammatory diseases in human lungs (Griese, 1999). The mucosal surfactant, which lines the respiratory tract, can engulf foreign organisms and particles, and alterations can be indicative of pathological conditions (Fig. 4; Milad & Morissette, 2021; Whitsett et al., 2010; Wright, 2005). Likewise, the higher expression and abundance of surfactant transcripts and proteins (SFTPA, SFTPB; **Chapter II, III**) in non-healthy harbour porpoises may indicate an increased turnover rate due to elevated pathogenic clearing or restricted respiratory function, ultimately affecting oxygen uptake (Milad & Morissette, 2021).

In general, the lungs are a well-oxygenated tissue due to the primary role in gas exchange and subsequent oxygen supply to dependant tissues, e.g., the brain, skeletal muscles and heart, via the blood circulation. As tissue gets damaged and, in the worst case, may be lost due to consumption by nematodes or persisting inflammation, the effective oxygen uptake can be reduced and oxygen capacity of the tissue may be lowered (Siebert et al., 2020; Ten Doeschate et al., 2017). While the lung tissue has been described to be resistant to lower oxygen levels (Wallace et al., 1974), resulting oxidative stress may exacerbate impacts caused by the pathological lesions observed in non-healthy harbour porpoises and further impede reduced oxygen capacity. Oxidative stress can form from reactive oxygen species (ROS) which are

produced naturally during metabolic processes or for chemotaxis signalling, but can also be generated during hypoxic conditions. In line with this, the lungs of the non-healthy cohort exhibited higher expression levels of protective antioxidants which hints at a disrupted redox homeostasis (**Chapter II, III**). The elevation of multiple features involved in glutathione metabolism points to an efficient protection from oxidative damage and highlights the importance of a strong antioxidative defence mechanism (Vázquez-Medina et al., 2006; Wilhelm Filho et al., 2002). Furthermore, antioxidative metabolites and lipids (plasmalogens) were significantly elevated (**Chapter III**). Plasmalogens are capable of binding free radicals, thereby reduce oxidative damage and alleviate inflammation (Brosche & Platt, 1998; Murphy, 2001). Although reduced plasmalogen levels have been associated with inflammatory diseases in humans (Paul et al., 2019), lungs of dolphins with recent experience of pneumonia showed elevated abundance levels similar to our results (Porrás-Gómez et al., 2025). This observation supports the potentially alleviating function of plasmalogens against inflammation and oxidative stress within the cetacean lung and warrants further research.

In combination, the observed immune responses in the lungs of harbour porpoises with severe lung nematode infestations point to worsening pulmonary health and are reminiscent of proinflammatory Th1/Th17 responses. This persistent immune activation may facilitate a self-sustaining cycle of chronic inflammation, promoting permanent tissue loss and reduced tissue function.

8.2.2 Metabolic reprogramming in the lung of non-healthy harbour porpoises

The lungs are often not considered as a metabolically active organ, but are indeed actively generating energy to ensure impeccable lung function (Liu & Summer, 2019). As the lungs are the first organ to receive the inhaled oxygen, it is reasonable to assume that energy is primarily generated via aerobic metabolic processes, such as the oxidative phosphorylation. However, it has been observed that the lungs employ multiple energy-generating pathways, ranging from glucose to fatty acid and branched amino acid metabolism, depending on condition (Iscra et al., 2002; Wallace et al., 1974). Another energy substrate in the lungs is lactate, which is utilised by the lungs for partial anaerobic glucose metabolism. This has been hypothesised to reduce the oxygen consumption of the lung itself, thereby prioritising optimised oxygen supply to distant and dependent organs (Fisher, 1984; Wallace et al., 1974). In non-healthy harbour porpoises suffering from a potentially impaired respiratory function due to vital tissue loss, enhanced lactate metabolism may be an alternative to compensate reduced oxygen uptake

and uphold oxygen distribution to sensitive tissues. While the results of the transcriptomic datasets do not support a higher lactate metabolism in non-healthy lungs (**Chapter II**), the results of a lactate dehydrogenase (LDH) assay (Fig. 5, see Additional Data 11.1) did show a significantly higher lactate utilisation in non-healthy compared to healthy lungs. Increasing lactate levels can become cytotoxic by lowering the pH value of the tissue environment (acidosis), thus has to be cleared swiftly to maintain favourable conditions. In support of this, the proteomic analysis revealed an upregulation of LDHB, which converts lactate to pyruvate (Fig. 6), indicating increased lactate conversion and clearance in non-healthy animals. Pyruvate can then enter the tricarboxylic acid cycle as substrate for energy generation (Fig. 6) or be utilised for protein synthesis.

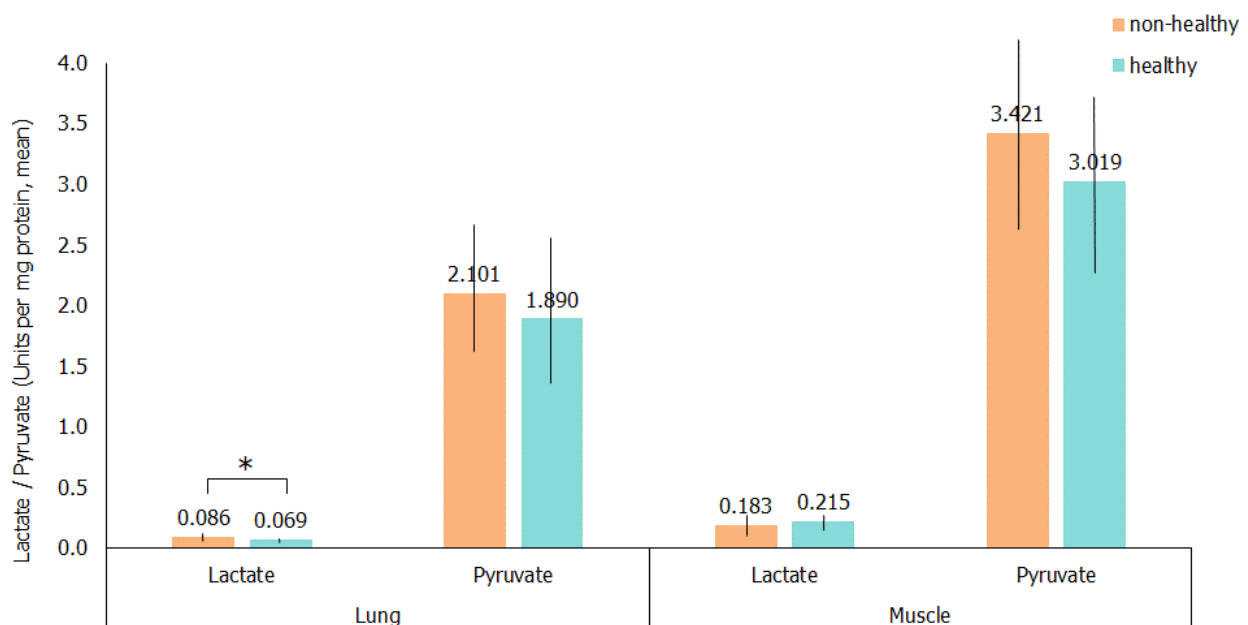


Figure 5 Lactate dehydrogenase (LDH) assay results. Depicted are the mean lactate concentrations (units per mg protein) in the lungs and skeletal muscles of non-healthy (n = 12) and healthy (n = 10) harbour porpoises. Concentration values are given above the columns. Two assays were conducted, following the protocols of Markert and Ursprung, 1962 (substrate: lactate) and Bergmayer et al., 1965 (substrate: pyruvate). Both protocols were adjusted in volume for measurement in 96-well plates and absorption was measured in 30 s-intervals for a total of 24 min (lactate) and 15 min (pyruvate) at constant 25 °C and 340 nm. Statistical significance was determined by Mann-Whitney U-tests, * indicates p-value < 0.05.

Higher glucose metabolism in non-healthy compared to healthy individuals, as indicated by the transcriptomic profiling (**Chapter II**), suggests that respiratory diseases substantially increase metabolic demand and consumption. The energy expenditure of acute immune activation, respiratory function and restorative processes is very costly (Dean et al., 2024; Fisher, 1984; Liu & Summer, 2019), and can lead to higher reliance on alternative metabolic pathways (Kivimäki et al., 2023). In line with this, lungs of non-healthy harbour porpoises exhibited elevated transcripts and proteins involved in the purine salvage pathway (e.g., HPRT1; **Chapter II, III**). An enhanced purine metabolism and recycling has also been observed in blubber

proteomes of seals after induced glucocorticoid stress and in several tissue samples of seals after fasting, thus may support ATP synthesis to meet energetic demand (Deyarmin et al., 2020; Soñanez-Organis et al., 2012).

Taken together, these alterations across major metabolic pathways in immune-challenged harbour porpoises indicate a progressive energy imbalance and highlight the need to better understand the energetic costs and management of immune responses and tissue function.

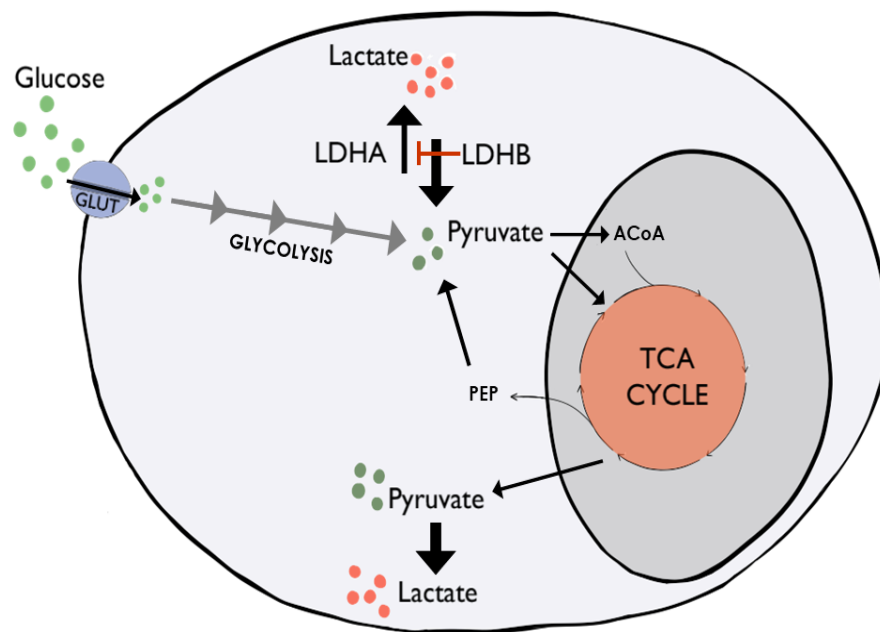


Figure 6 Glucose metabolism and the function of lactate and lactate dehydrogenase (LDH). Depicted is a part of the cellular glucose metabolism. Under hypoxic conditions, glycolysis is elevated and oxidative phosphorylation is impaired. Pyruvate served as substrate for LDHA (the more anaerobic form) to generate lactate which is then metabolised to generate ATP. In a reversed reaction, the preferred aerobic LDH form, LDHB, metabolises lactate to pyruvate, a reaction that clears cytotoxic accumulation of lactate within the cell and supports the TCA cycle. GLUT: glucose transporter, ACoA: acetyl-coenzyme A, PEP: phosphoenolpyruvate. Graphic by Eda Dönmez

8.3 Systemic inflammation beyond the lungs: the muscles

8.3.1 Systemic consequences of an impaired pulmonary system

An impaired pulmonary function impairs the oxygen cascade at its primary step by reducing efficient gas exchange and optimal lung tissue function. As a result, oxygen concentration in the blood is lowered and the oxygen delivery to receiving organs is limited. Particularly, organs with a high metabolic demand and an essential function are sensitive to reduced oxygen availability.

Several studies on human lung diseases associated with a reduced respiratory function (such as COPD, pneumonia, respiratory distress syndrome, and fibrosis), have reported significant comorbidities and multiorgan dysfunction, including effects on the skeletal muscles (Mou et al., 2024; Noguera et al., 2001). These observations highlight that an impaired respiratory function can result not only in localised, but also in systemic consequences.

Our findings indicate a chronic inflammatory state in the lungs of non-healthy harbour porpoises which likely increases energy demand and promotes persistent tissue loss. Importantly, these effects are not confined to the respiratory system. The muscles of non-healthy harbour porpoises exhibited a similar dysregulation of biological processes as observed in the lung, strengthening the assumption of systemic inflammation in non-healthy individuals (**Chapter I, II, III**). Such a systemic response can be explained by reduced respiratory function and excessive immune activation originating in the lung. Similarly, preliminary results of other comparative studies in harbour porpoises and dolphins with lung disease noted a negative impact on respiratory function (approx. 40% decline in airflow with pneumonia, Borque-Espinosa et al., 2020; reduced tidal volume with lung abscess, Kastelein et al., 1997a), although the sample size of these studies was limited to few individuals. Continuing from the respiratory system, the inflammation can extend into the vasculature and subsequently manifest within other organs downstream of the oxygen cascade (Bernardo et al., 2015; Sinden & Stockley, 2010).

Across both lungs and muscles, several transcripts, proteins, metabolites, and lipids showed a similar dysregulation in non-healthy harbour porpoises (**Chapter II, III**) that may represent downstream signalling or a shared response of the two tissues. Among these features, several had a role in oxidative stress responses and were associated with inflammatory processes (Fig. II.3, Fig. III.6), supporting a potentially systemic response to decreased oxygen delivery and exacerbating inflammation. Two overlapping features that may be of particular interest were Protein Dispatched Homolog 1 (DISP1) and the metabolite isovalerylcarnitine (**Chapter III**). Both were significantly elevated in the lungs and muscles of non-healthy individuals (Fig. III.6). The potential of DISP1 to indicate respiratory disease is not only supported by its role in hedgehog signalling, which is implemented in COPD pathogenesis and pulmonary fibrosis, it has also already been proposed as a biomarker for COPD in humans (Ancel et al., 2020; Cigna et al., 2012). Likewise, isovalerylcarnitine possesses a distinct abundance pattern in pneumonia and respiratory distress syndrome in humans and has been tested as prognostic disease progression tool (den Hartog et al., 2024; Suber et al., 2023). Moreover, isovalerylcarnitine is

produced as a by-product during incomplete metabolism of the branched chain amino acid leucine (McCalley et al., 2019; Tanaka et al., 1966), for which the decreased abundance of leucine and isoleucine in the muscle of non-healthy harbour porpoises may be further proof (**Chapter III**). Additionally, isovalerylcarnitine can also be produced from carnitine and the generally toxic isovaleric acid (McCalley et al., 2019; Tanaka et al., 1966). Whales rely heavily on lipid metabolism and also possess specialised lipids that do not get metabolised and serve important functions (e.g., acoustic fats, insulating blubber), in which isovaleric acid is highly concentrated (Koopman, 2018). Next to these fatty tissue, muscles of harbour porpoises also seem to exhibit higher concentrations of isovaleric acid compared to other non-fatty tissues, such as the lung in which it is not present (Arregui et al., 2020; Koopman et al., 2003; Lovern, 1934). Therefore, higher abundance of isovalerylcarnitine in the lung may hint at progressive disease conditions, as similarly observed in humans. Whether the observed accumulation in the muscles may be neglectable or whether a certain threshold exists within the tissue, beyond which isovalerylcarnitine accumulation is no longer tolerable, has to be elucidated further.

The muscles of non-healthy harbour porpoises may also respond to systemic inflammation and other associated effects through the upregulation of non-coding RNAs (ncRNAs), which were identified via a *de novo* transcriptome assembly (**Chapter II**). Although ncRNAs were previously thought to be non-functional, mounting evidence in mice and humans indicates that they can modulate adaptive immune responses in a spatiotemporal manner (Grammatikakis & Lal, 2022; Mowel et al., 2018). However, whether the significantly dysregulated ncRNAs identified in this study mediate immunomodulatory or other protective effects in response to a systemic inflammation remains to be investigated in further *in vitro* studies.

Valuable insights and supporting results could have been obtained by including data sets of blood samples from non-healthy and healthy harbour porpoises. However, due to the limited possibility to acquire blood samples from a sufficient sample size in both cohorts, such a comparison was not feasible. Also, due to non-healthy harbour porpoises often suffering not only from respiratory diseases, but concurrently from a multitude of diseases and health impacts, the dysregulation cannot with full certainty be attributed solely to the observed pathological lung lesions. Still, the overlaps in dysregulated features in both tissues highlights the systemic inflammatory state and coordinated molecular response in non-healthy harbour porpoises.

8.3.2 Stress and metabolic responses in the musculature

Increased immune activation within the whole body can also exacerbate low oxygen conditions and be a source for elevated oxidative stress and hypoxemia (Couillard & Prefaut, 2005), adding to the cumulative stress experience. Similarly, muscles of non-healthy harbour porpoises showed significant elevation of various antioxidative processes (**Chapter I, II, III**), underlining exceeding levels of oxidative stress. While marine mammal muscles possess higher baseline antioxidant levels compared to terrestrial mammals (Allen & Vázquez-Medina, 2019; García-Castañeda et al., 2017; Vázquez-Medina et al., 2006), muscles are more likely to accumulate cellular damage as a post-mitotic tissue (Frontera & Ochala, 2015; Rom & Reznick, 2016). Since systemic inflammation can trigger reactive oxygen species production in the skeletal muscles (Reid & Li, 2001), harbour porpoises may enhance the deposition of antioxidative and anti-inflammatory plasmalogen lipids within the tissue (**Chapter III**). The simultaneously enhanced levels of plasmalogens in the lungs and muscles of non-healthy individuals hint at an effective strategy in cetaceans and additional studies may foster deeper insights into their potential role against accumulating oxidative damage and inflammation. Several of the elevated oxidative stress responses observed in the muscles of non-healthy harbour porpoises have been well-studied in cetaceans, including glutathione, peroxiredoxins and superoxide dismutases (Fig. 7, Allen & Vázquez-Medina, 2019; Cantú-Medellín et al., 2011; García-Castañeda et al., 2017; Tian et al., 2021b). However, the prominent elevation of features involved in glutathione metabolism (**Chapter I, II, III**) highlights the significance and potency of this protective system in cetaceans (García-Castañeda et al., 2017; Tian et al., 2019, 2021a; Vázquez-Medina et al., 2007). Glutathione exists in two states, namely reduced (GSH) and oxidised (GSSG), with GSH acting as radical scavenger (Owen & Butterfield, 2010). While we performed an assay to analyse GSH concentrations within the tissue, no significant and reliable results could be obtained (see Additional Data 11.2). Likewise, despite being detected in the metabolomic analyses, no significant difference between conditions was detected for GSH or GSSG (**Chapter III**). As oxidants are very fleeting and readily oxidise from the tissue or protein samples with repeated exposure to atmospheric oxygen (e.g., during subsampling and freeze-thaw cycles), a considerable amount may already have been lost. Still, the detection of elevated glutathione-associated transcripts (e.g., glutathione-S-transferase 3 GSTT3, cystathionine- γ -lyase CTH, **Chapter I, II**) and proteins, such as glutathione-S-transferase, glyoxalase GLO1, and peroxiredoxins PRDX2 and PRDX6 (**Chapter III**), suggests that cetaceans rely heavily on this antioxidative system which has also evolved to be more efficient in marine mammals (Allen et al., 2019; Cantú-Medellín et al., 2011; Tian et al., 2019, 2021a). In addition to the oxidative

stress that may be exacerbated by lung-associated inflammation, hypoxia is a common factor or symptom of inflammatory disease (Vohwinkel et al., 2015). This may be reflected by the observation of enhanced stress and hypoxia-associated signalling in the muscle tissue (FoxO, HIF1; **Chapter II**). Although cetaceans experience hypoxic conditions on a regular basis and have adapted to these states by upregulation and optimisation of hypoxia-alleviating processes (Allen et al., 2019; Ramirez et al., 2007; Zhu et al., 2018), the upregulation in non-healthy compared to healthy porpoises hints at a higher hypoxic challenge. With lung gas exchange and function potentially reduced due to persistent inflammation and subsequent damage, oxygen supply would be reduced to the muscles, thus likely results in an oxygen deficit. For harbour porpoises, which possess limited oxygen storage capacity due to their body size (Spitz et al., 2012), this may lead to deleterious effects on diving capability and ultimately to a higher risk of inadequate diving, foraging, and reduced fitness. In addition, marine mammals can “plan” their dives and adjust their diving response accordingly (Arranz et al., 2018; Elmegaard et al., 2016, 2019; Fahlman et al., 2024). However, harbour porpoises are constantly exposed to anthropogenic disturbances (Halpern et al., 2008, 2015). Disrupted or suddenly altered behaviour to avoid stressors, e.g., by deeper diving or rapid fleeing (Elmegaard et al., 2023; Frankish et al., 2023), may aggravate the disproportion between required and available oxygen in immune-challenged and stressed animals.

Next to the generally high energy demand of the muscle for its basal functions in locomotion and thermogenesis, stress responses and immune activation pose as extraordinary energetic challenges (Lochmiller & Deerenberg, 2000). Due to their aquatic adaptations, obvious and continuous impacts on energy consumption, such as hypoxia and oxidative stress, have been studied intensely in marine mammals (Fair & Becker, 2000; Horscroft & Murray, 2014; Khudyakov et al., 2015b). In whales, energy is preferably produced from lipids (Chicco et al., 2014; Deros et al., 2022; Kanatous et al., 2008; Sierra et al., 2015; Velten et al., 2013) and under normal conditions, the musculature in mammals requires up to 30% of available energy (De Lange et al., 2007). With the muscle mass of harbour porpoises being comparable to that of humans (approx. 30-40%; Frontera & Ochala, 2015; McLellan et al., 2002), their musculature’s energy expenditure is expensive. Hence, a small reduction in efficiency of lung function and herewith, sufficient oxygen supply to the muscles for energy production, can manifest in marked metabolic consequences (Borque-Espinosa et al., 2020; Tsang et al., 2025).

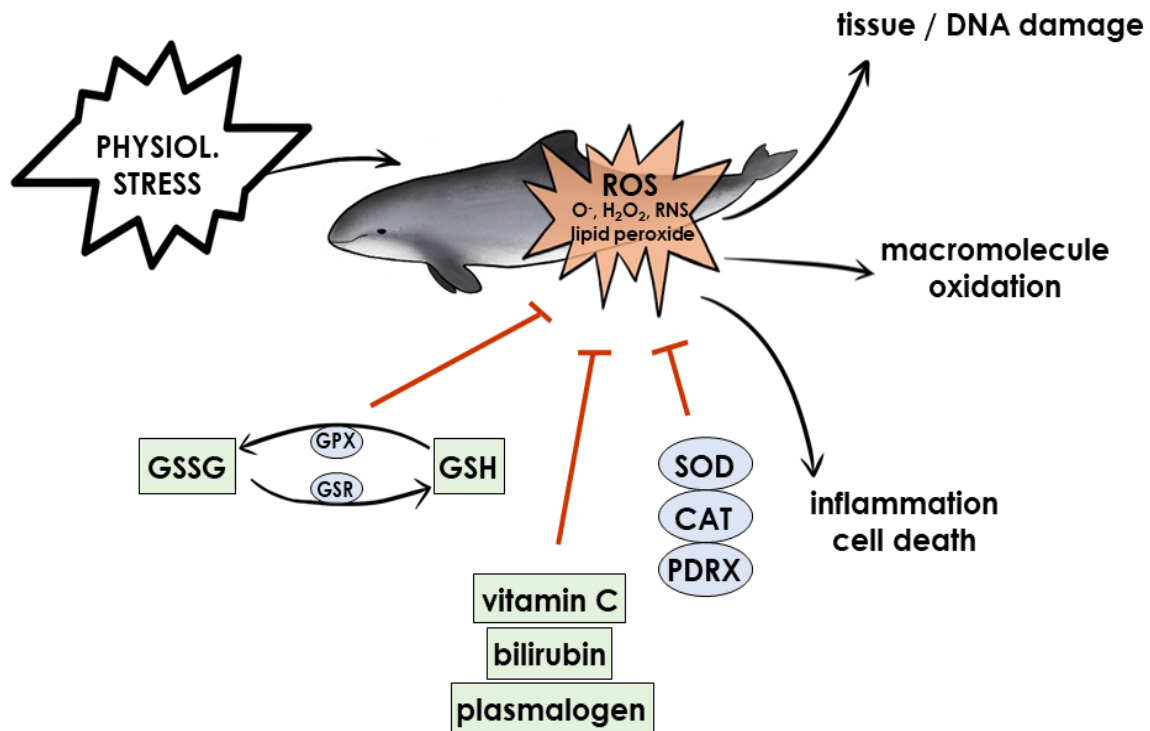


Figure 7 Schematic of the antioxidant system. Shown are the main components of the extensive cellular oxidative stress response which includes glutathione-associated processes (GPX, GSR), superoxide dismutases (SOD), catalase (CAT) and peroxiredoxins (PRDX). All of these enzymes primarily detoxify reactive oxygen species (ROS), reactive nitrogen species (RNS) and other potentially harmful oxidants or free radicals. The accumulation of deleterious ROS/RNS can cause cell or DNA damage and cytotoxicity, interfere with biological processes, and cause inflammation or cell death. Enzymes are represented as blue ellipses, metabolites and lipids are displayed by green rectangles. Illustration by Eda Dönmez

A similar situation is illustrated by our results. Muscles of harbour porpoises suffering from respiratory disease and parasites exhibited dysregulation of glucose metabolism and lipid metabolism (**Chapter I, II, III**). Transcriptomic and proteomic profiling of the muscles of non-healthy harbour porpoises suggested an enhanced tolerance for aerobic lipid metabolism and stress-associated activation of this energetic process (**Chapter I, II**), which has previously been described in several tissues of other marine mammal species under stress (Derosus et al., 2021; Khudyakov et al., 2015a, 2017; Tian et al., 2017; Torres-Velarde et al., 2021). While high reliance on fatty acid metabolism was implied by upregulation of a plethora of transcripts and proteins involved in lipid energy production and homeostasis (**Chapter I, II, III**), the main lipid metabolic pathway, β -oxidation, did not show similar elevation (**Chapter III**). As the diet of harbour porpoises consists of high-fat prey, and toothed whales have evolutionary lost the ability for ketogenesis (Wolfgang et al., 2021), β -oxidation represents the primary pathway for energy generation. If this option is either unavailable or impaired, these animals may be compelled to resort to alternative pathways to cover the increasing energetic requirements associated with systemic immune activation.

Moreover, muscles of non-healthy harbour porpoises exhibited indicators of higher anaerobic metabolism, indicating exhaustion of available muscle oxygen stores, but persisting energetic demand (**Chapter I, II, III**; Champagne et al., 2012; Nedvedova et al., 2018). Induction of glycolysis for energy production is part of the mammalian response to hypoxia and inflammation (Soto-Herederó et al., 2020). Skeletal muscles switch to anaerobic energy production when stored oxygen (e.g., in myoglobin) is depleted which enables them to perform short-term, high-intensity exercise. With the muscles consuming a substantial amount of energy and oxygen for locomotion (Davis, 2014; Pabst, 1993), and considering that harbour porpoises due to their high metabolic field rate are forced to constantly forage (Rojano-Donãte et al., 2018), reduced oxygen supply and inflammatory spill-over from the lungs may lead to a significant energy deficit (Lochmiller & Deerenberg, 2000; Sinden & Stockley, 2010). Though increased glycolytic rates have already been confirmed in persistently stressed marine mammals (Champagne et al., 2012; Park et al., 2015; Tian et al., 2017; Torres-Velarde et al., 2021), and muscles of non-healthy harbour porpoises exhibited elevation of anaerobic metabolism-associated transcripts (**Chapter I, II**), results of the LDH assay did not show a significant difference in lactate or pyruvate utilisation between health conditions (see Additional Data 11.1). One possible reason may be the high anaerobic buffering capacity of harbour porpoise muscles (Noren et al., 2014) which could aid with swift lactate clearance, irrespective of physical condition.

Interestingly, uncoupling protein 3 (UCP3) was identified in the transcriptomic analyses which showed reduced expression levels in non-healthy animals (**Chapter I, II**). UCP3 has not yet been detected in cetaceans. Studies have described cetacean UCP1 which is pseudogenised, therefore without enzymatic function (Gaudry et al., 2017; Zhou et al., 2022). UCPs are involved in uncoupling of the oxidative phosphorylation, which promotes proton leakage and mitochondrial dysfunction (Levraut et al., 2003; Zhou et al., 2022). Thus, a reduction in expression may enhance efficient energy utilisation and generation, although it is unclear whether UCP3 is pseudogenised as well, or what function it inherits, if functional.

8.3.3 Muscle atrophy and regenerative capacity of non-healthy harbour porpoises

Skeletal muscles can swiftly adjust to external changes, for which they possess a high tissue turnover rate (Frontera & Ochala, 2015; Howard et al., 2020). Therefore, muscle degradation and regeneration are naturally reoccurring processes that aid with fast replacement of injured tissue, enable muscle growth and recovery after strenuous exercise, as well as clearance of cellular debris (Call & Nichenko, 2020; Xia et al., 2021). As energy expenditure becomes more

aberrant with progressing systemic inflammation, non-healthy harbour porpoises may be forced to metabolise muscle tissue to cover energetic demands (Wolfe, 2006). Consistent with this, the muscles of non-healthy harbour porpoises showed high expression of transcripts indicative of muscle loss and autophagic flux (TRIM63, SQSTM1; **Chapter I, II**; Rom and Reznik, 2016; Thoma and Lightfoot, 2018; Puissant et al., 2012). In humans and mice, muscle loss (atrophy) has been a commonly observed comorbidity of lung diseases (Henrot et al., 2023; Plant et al., 2010; Puig-Vilanova et al., 2015), suggesting that the same may also be true for harbour porpoises suffering from similar conditions. Additionally, atrophy can alter fibre type composition and with this, muscle characteristics (Maltais et al., 2014; Passey et al., 2016), which may in turn affect the diving and foraging abilities of harbour porpoises with declining pulmonary health (Dejong et al., 2005; Langhans et al., 2014; Sin & Man, 2006). Furthermore, muscle tissue can serve as a final energy reserve (McLellan et al., 2002; Rojano-Doñate et al., 2024). Therefore, accelerated muscle loss may indicate starvation in non-healthy harbour porpoises which are often found in an emaciated state (IJsseldijk et al., 2022; Kastelein et al., 2019; Neimanis et al., 2022). Taken together with the observed dysregulation of the main metabolic pathways, the indicated muscle loss may worsen a potential energy shortage by impairing muscle function and intensifying muscle catabolism to ultimately supply amino acids for gluconeogenesis (Call & Nichenko, 2020; Whiteman et al., 2017). In line with this finding, the metabolomic analysis of the muscles of non-healthy harbour porpoises revealed higher abundance of proline, isoleucine and leucine (**Chapter III**), which can be utilised for energy generation (Jagoe & Engelen, 2003). Proline can be catabolised to subsequently provide α -ketoglutarate, and isoleucine and leucine can serve as intermediate substrates from which acetyl-coA can be generated. These substrates can be then introduced into the tricarboxylic acid cycle. Interestingly, diseased dolphins showed a similar dysregulation of proline, isoleucine and leucine in their plasma when compared to healthy individuals (Derous et al., 2022), further strengthening our findings.

The experience of cumulative stressors (e.g., nutritional imbalance, oxidative stress) can exacerbate the state of atrophying muscles and cause significant harm by inhibiting or interfering with translation processes (Lian et al., 2022). Likewise, ribosomal processes are usually among the first processes that are reduced under stress due to their high energetic costs (Shah et al., 2013). Contrary to these findings, protein translation and ribosome biogenesis were elevated in non-healthy harbour porpoises (**Chapter I, II**). Taken together with the upregulation of transcripts and proteins involved in wound healing, tissue regeneration and muscle function (**Chapter I, II, III**), this may imply a higher regenerative activity in the

muscles of non-healthy harbour porpoises. Additionally, the higher expression of transcripts employed at cell cycle check points indicted increased control and minimisation of misfolded proteins or damaged DNA, thereby aiding in tissue maintenance (Call & Nichenko, 2020; Frontera & Ochala, 2015; Howard et al., 2020). Increasing processes involved in regeneration and cell integrity, such as ribosome biogenesis, translation and damage control mechanisms, while degradational flux is high may enable swift replacement of damaged cells, ensuring tissue integration and function. A similar regulation pattern as a response upon injury or damage has been observed in other mammals as well (Nixon et al., 2016; Xia et al., 2021; Yadava et al., 2016). Still, the high energetic costs to simultaneously uphold these processes can significantly impact muscle integrity and function and the overall energy balance of non-healthy harbour porpoises. If these animals are hindered to dive and forage sufficiently, they may increasingly suffer from starvation and weakened body condition. The resulting exacerbation of muscle atrophy to sustain such energy-intensive processes may aggravate the incompetency of the porpoises to maintain their health and avoid stressors. As several recent studies observed rapid fluking, spontaneous deeper diving and prolonged, fast acceleration of swimming speed to flee from underwater-radiated noise (Elmegaard et al., 2023; Frankish et al., 2023), an ubiquitously encountered stress factor (e.g., due to seismic surveys, fisheries, shipping), significant loss of muscle tissue and impairment of its function may consequently lead to a higher cumulative stress exposure, and decreased resilience and survival.

8.4 Towards biomarkers for health assessment in free-ranging harbour porpoises

Biomarkers can be a helpful tool to remotely and minimally or non-invasively monitor body condition (Kershaw et al., 2017), reproduction (Melica et al., 2021), chronic stress (Maia et al., 2025), contaminant burden and health of cetaceans (Lehnert et al., 2019; Simond et al., 2020). As these insights are valuable for population biology or conservation, the establishment of stable biomarkers has been the focus of a plethora of studies. In general, a biomarker is used to identify pathological or altered physiological states and often is a molecule (e.g., gene, transcript, metabolite, hormone) that occurs naturally within the animal (Aronson & Ferner, 2017). For cetaceans, many biomarker studies focus on more easily accessible tissues. For example, skin, blubber and muscle can be obtained via biopsies from live animals (Noren & Mocklin, 2012; Van Cise et al., 2024), while blood samples are often taken during capture-

release handling or voluntary veterinary inspection (Unal et al., 2018). With advancing methods and the goal towards minimising physical harm to wild animals, studies have also analysed breath exhale (blow) samples for suitable health biomarkers (Godard-Codding & Fossi, 2018). Another approach is the use of tissues taken during post-mortem examinations (Hamano et al., 2023; IJsseldijk et al., 2019), which was also employed for the here presented studies.

While the integration of multiple omics data sets (e.g., transcriptomics, proteomics, microbiomes or genomics, among others) for biomarker identification is a relatively recent method, its application has increased in recent years with technological advances (Bowerman et al., 2020; Harriott et al., 2025; Ivanova et al., 2024; Konigsberg et al., 2021). Integration of omics data of the same tissue from the same individuals can not only give a more holistic overview of regulated responses and activated mechanisms (Lee et al., 2019), but also strengthen the results of single omics layers (Graw et al., 2021). Therefore, we aimed to identify a signature of potential health assessment markers in the lungs and muscles by integrating proteomic, metabolomic and lipidomic data from the same six non-healthy and six healthy harbour porpoises (**Chapter III**). Although there is a growing abundance of tools to integrate omics data, here, the “DIABLO” tool of mixOmics was used which was specifically developed for identification of biomarkers (Harriott et al., 2025; Singh et al., 2019). Among the 18 features identified for each tissue, two highly correlated signatures stood out, which were promising in successfully discriminating between conditions. In the lung, the signature comprised protein SLC25A4, metabolite O-phosphoethanolamine and lipid TG O-16:0_16:0_20:4 (Fig. III.5; Table III.5). Within this interacting network SLC25A4 enables the transport of ADP:ATP (Cl  men  on et al., 2013), while triglycerol lipids, such as TG O-16:0_16:0_20:4, are commonly used for energy metabolism. On the other hand, O-phosphoethanolamine has been associated with higher tolerance of irritating ozone in lungs of mice, supporting a protective function (Smith et al., 2023). With our previous results implying that the highest dysregulation is caused within immune responses and metabolic processes (**Chapter II, III**), this marker signature may be suitable to monitor lung-associated disease and inflammation in free-ranging harbour porpoises.

The identified signature in the muscle consisted of three tightly correlated features, namely the protein SPEG, the lipid BMP 18:1_22_6 and the metabolite pipecolic acid, which additionally showed positive correlation with two plasmalogens (PE P-18:0_18:1, PE P-17:0_22:6; Fig. III.6; Table III.5). The features in this signature are involved in muscle function (SPEG; Campbell et al., 2021), cellular trafficking (BMP 18:1_22_6; Showalter et al., 2020) and have been observed in association with smoking-induced oxidative stress (pipecolic acid; Dalazen et

al., 2014; Shen et al., 2023). The positive correlation between pipecolic acid with two anti-inflammatory and antioxidative plasmalogens may point to an adaptive measure to protect the tissue upon increased oxidative stress (Brosche & Platt, 1998; Murphy, 2001; Paul et al., 2019). Similar to the signature in the lung, these features cover most of the affected processes identified in the muscles of non-healthy harbour porpoises (**Chapter I, II, III**), thus may qualify as an applicable biomarker panel to observe potential pathological consequences caused by the pathological lung. The use of a panel of markers, instead of one single molecule, has also been deemed more reliable, as multiple, simultaneously tested molecules add predictive power and the combination of molecules across several systemic layers strengthens the significance of the results (Smith et al., 2021).

While the predictive power in this study is restricted by the relatively small sample size, the identification of selected features for further verification in larger cohorts and in functional studies is a crucial step to support health monitoring in free-ranging cetaceans.

9 Conclusions and Perspectives

This integrative work on harbour porpoises presents an important step toward understanding how free-ranging cetaceans cope with persistent pathological impacts and subsequent immune challenge at the whole-organism level. By linking changes in the pathological lung with corresponding muscles of non-healthy harbour porpoises, it highlights that respiratory disease in marine mammals is not a localised issue of the lung, but a systemic condition that reshapes pathophysiological processes, metabolism and tissue maintenance. These broader perspectives underline how cetacean pulmonary health balances dynamics between damage, compensation and functional decline, which result in direct implications for diving performance, survival and ultimately also concern population biology.

The identification of conserved immune patterns between harbour porpoises and other mammals suffering from nematode infestations or other lung diseases suggests that comparative, cross-species approaches can be useful to interpret findings of cetaceans, a clade for which molecular reference data oftentimes is scarce. Advances in technology, such as omics tools, combined with increasing cost-efficiency, broadens the spectrum of possible cetacean studies, leading to increased data generation and therefore, facilitates more in-depth analyses.

The prominent roles of oxidative stress, altered energy metabolism and finely tuned regeneration–degradation cycles in the muscles point toward a new research focus on energetic trade-offs. Future work is necessary to assess how much additional energetic burden diseased animals can sustain before critical functions, such as foraging, migration or reproduction, are compromised. This will be particularly relevant as ocean conditions change, and cumulative anthropogenic and environmental pressures from underwater-radiated noise, contaminants, and climate change intensify, potentially narrowing the margin for compensation.

The candidate biomarker panels identified in the lungs and muscles enable future application in which molecular diagnostics can be integrated into non- to minimally invasive health assessment of free-ranging cetaceans. While these markers need to be validated in larger and more diverse cohorts, they could allow for early detection of disease and evaluation of rehabilitation success. In a broader conservation context, molecular markers of systemic stress and compromised function may help to quantify the impact of anthropogenic stressors and support evidence-based management decisions.

Overall, this study highlights how prevalent respiratory disease in harbour porpoises accounts for chronic, multifactorial stress in an increasingly human-impacted ocean. The current findings provide both a conceptual and a methodological framework for future work that will deepen our understanding of cetacean physiology, enable new ways for identification of molecular health indicators and underscore the implications of individual pathology on population biology.

10 References

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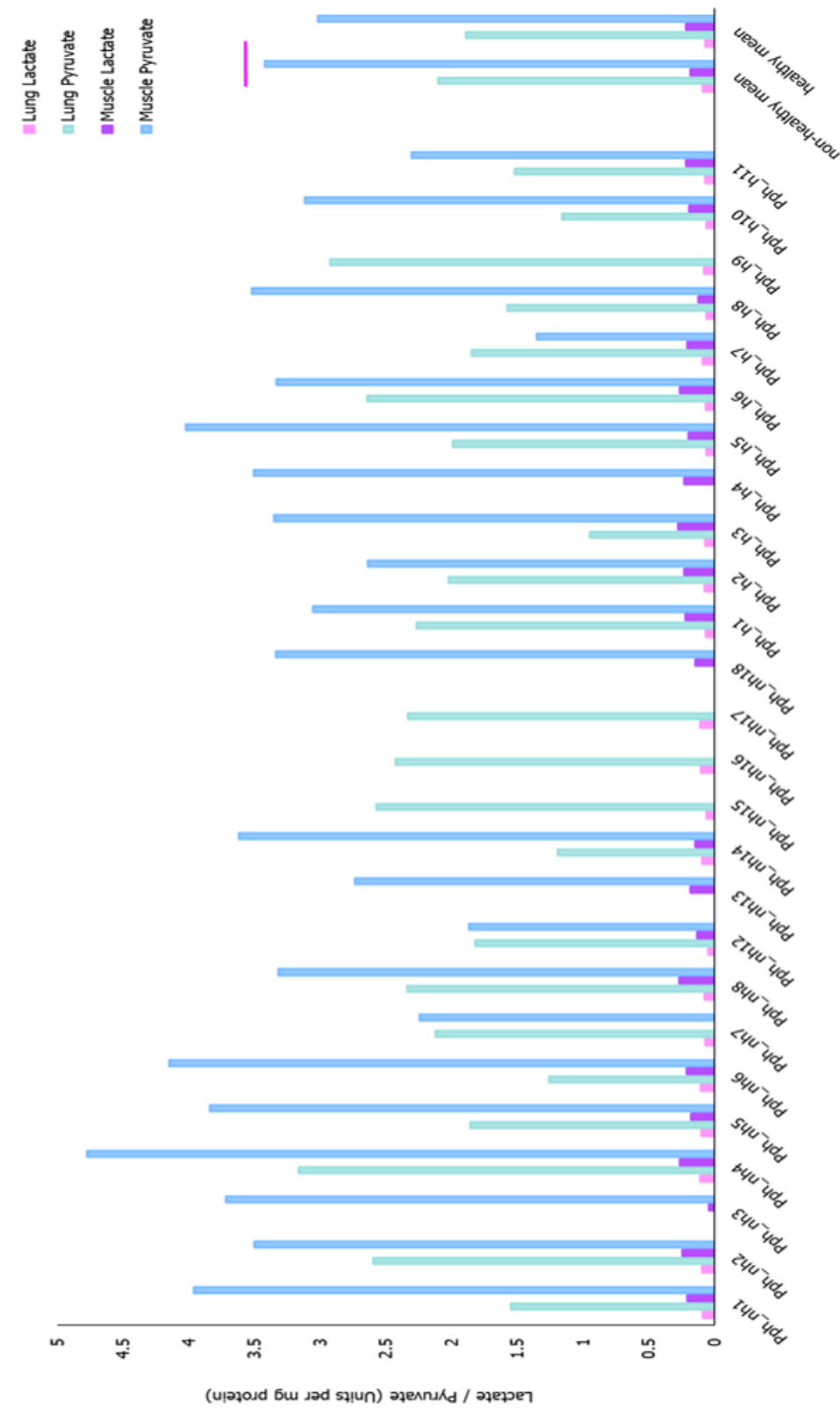
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11 Additional data

11.1 LDH-Assay

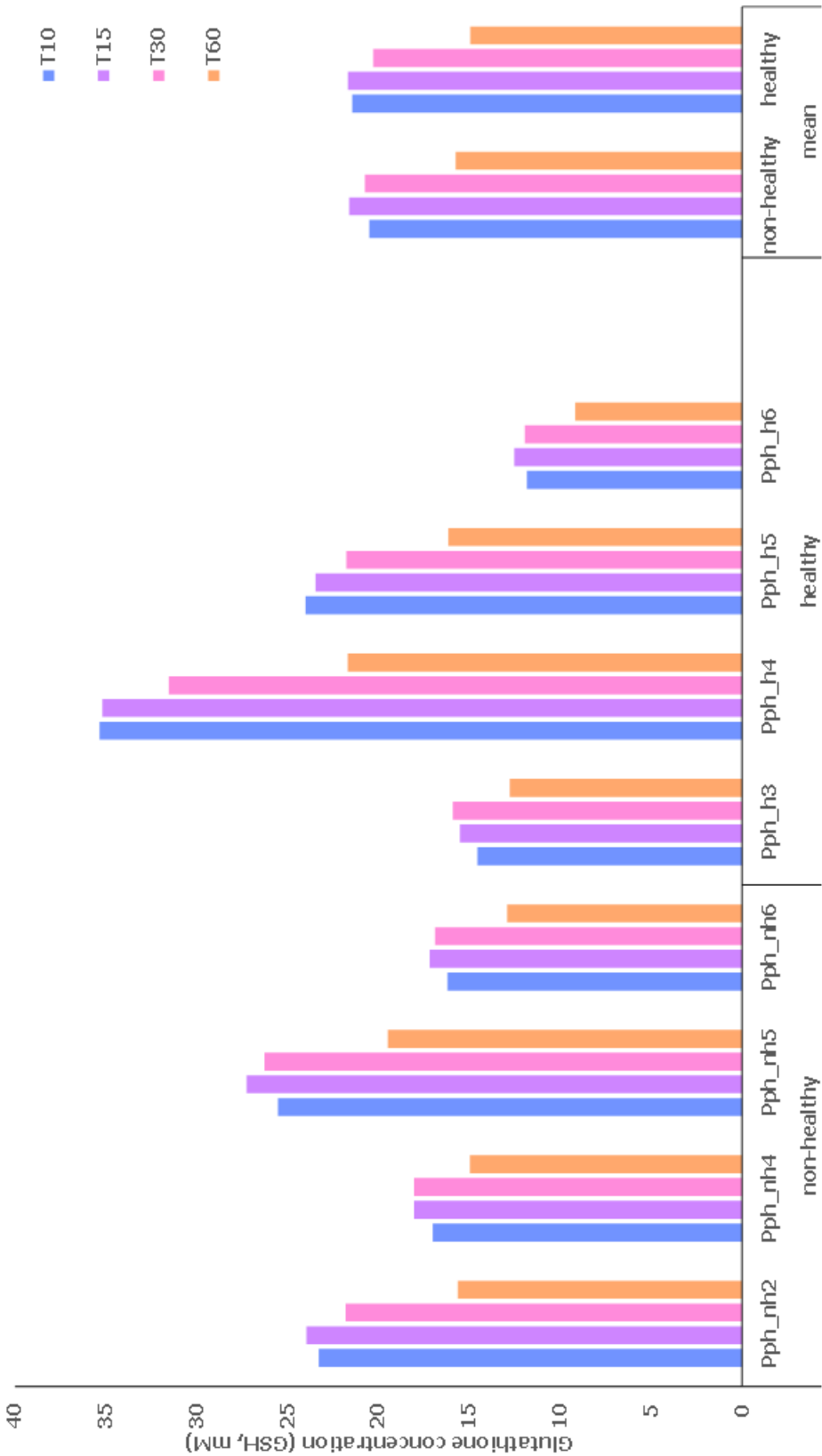


Additional Figure 1. Results of the LDH Assay. Shown are the measured units of lactate (pink and violet) and pyruvate (turquoise and blue) per mg protein for the individual porpoises and the mean of the cohorts. Protein was extracted from the lungs and the muscles and the assays were performed following the protocols of Bergmayer et al., 1965 (pyruvate) and Markert and Ursprung, 1962 (lactate). Both protocols were adjusted in volume for 96 well plates, and absorption was measured with a plate reader at 340 nm in 30 s-intervals for a total of 24 minutes (lactate) and 15 min (pyruvate). Statistical significance was determined with a Mann-Whitney U test. Only the results of the lactate assay of the lungs from non-healthy compared to healthy harbour porpoises showed statistical significance (p-value < 0.05).

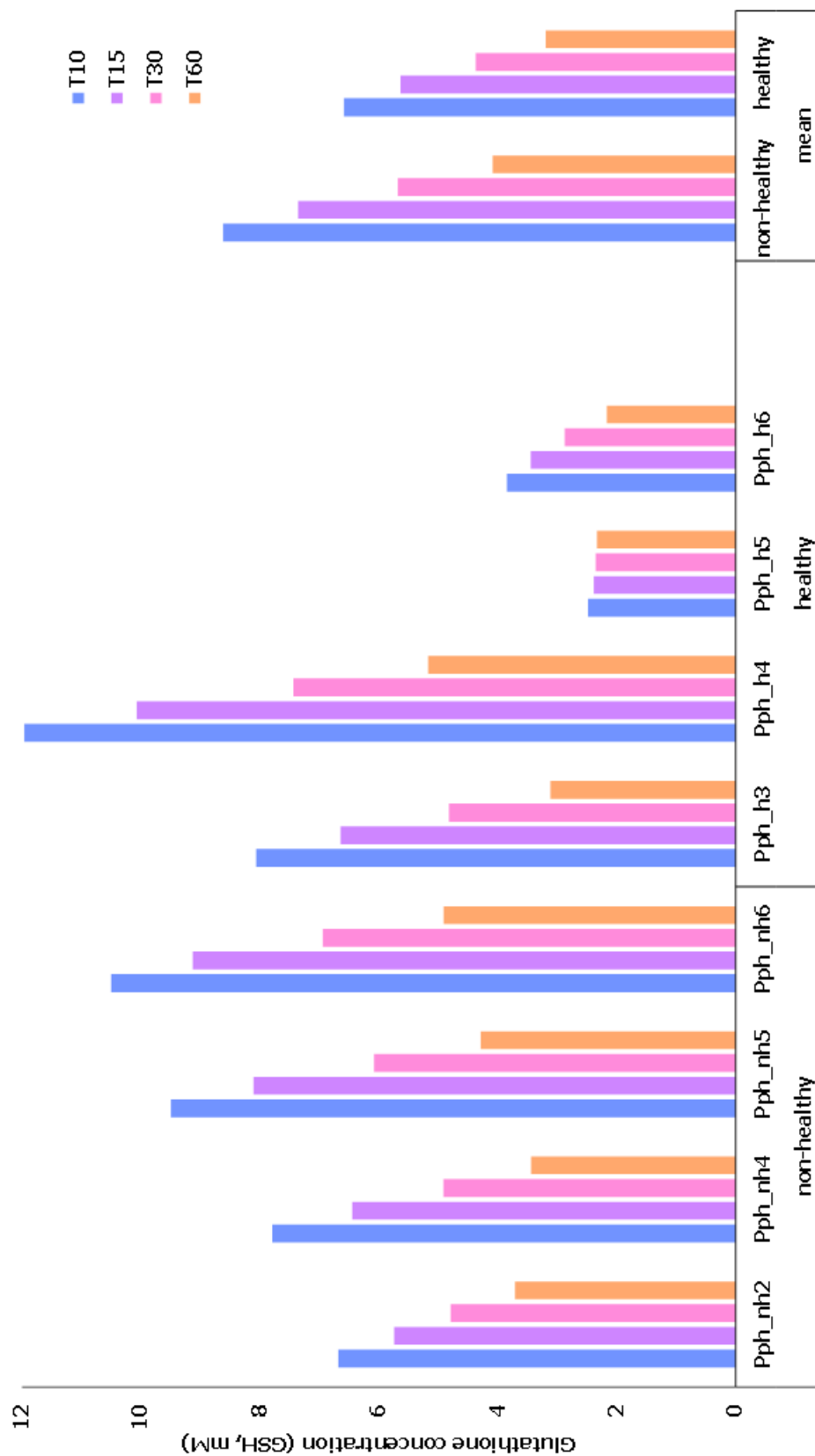
Additional Table 1. Results of the LDH assay. Listed are the measured units per mg protein (U per mg) of lactate and pyruvate in the lungs and muscles of the individual porpoises. The mean of the cohorts and the standard deviation (Std) are also given. nh: non-healthy individuals, h: healthy individuals.

Individual	Lung		Muscle	
	Lactate (U per mg)	Pyruvate (U per mg)	Lactate (U per mg)	Pyruvate (U per mg)
Pph_nh1	0.0861	1.5465	0.2077	3.9591
Pph_nh2	0.0916	2.5972	0.2449	3.5029
Pph_nh3			0.0426	3.7165
Pph_nh4	0.1046	3.1615	0.2641	4.7726
Pph_nh5	0.0974	1.8576	0.1759	3.8378
Pph_nh6	0.1000	1.2551	0.2079	4.1476
Pph_nh7	0.0705	2.1226		2.2439
Pph_nh8	0.0763	2.3349	0.2671	3.3184
Pph_nh12	0.0470	1.8194	0.1296	1.8668
Pph_nh13			0.1818	2.7356
Pph_nh14	0.0909	1.1905	0.1435	3.6163
Pph_nh15	0.0589	2.5722		
Pph_nh16	0.1006	2.4245		
Pph_nh17	0.1077	2.3335		
Pph_nh18			0.1458	3.3381
Pph_h1	0.0625	2.2650	0.2211	3.0547
Pph_h2	0.0720	2.0224	0.2296	2.6368
Pph_h3	0.0713	0.9492	0.2763	3.3487
Pph_h4			0.2301	3.5069
Pph_h5	0.0602	1.9881	0.1964	4.0237
Pph_h6	0.0659	2.6420	0.2641	3.3342
Pph_h7	0.0882	1.8484	0.2051	1.3487
Pph_h8	0.0623	1.5768	0.1196	3.5174
Pph_h9	0.0810	2.9255		
Pph_h10	0.0597	1.1589	0.1915	3.1180
Pph_h11	0.0716	1.5201	0.2159	2.3032
mean non-healthy				
mean healthy	0.0860	2.1013	0.1828	3.4213
Std non-healthy	0.0695	1.8896	0.2150	3.0192
Std healthy	0.0094	0.6202	0.0431	0.7574

11.2 Glutathione-Assay (GSH)



Additional Figure 2. Glutathione-Assay of the lung. The glutathione assay has been performed following the instructions of the used assay kit ab205811 (fluorometric) by abcam. Only GSH was measured. Depicted are the measured concentrations of glutathione (GSH, in mM) in the lungs of non-healthy and healthy muscles. Individual concentrations and mean values for both cohorts are given. Concentration has been measured at multiple timepoints after incubation and start of absorption measurement (10 min, T10; 15 min, T15; 30 min, T30 and 60 min, T60). Statistical significance was determined with a Mann-Whitney U test. No timepoint comparison between the two conditions was significantly different.



Additional Figure 3. Glutathione-Assay of the muscle. The instructions of the used assay kit ab205811 (fluorometric) by abcam were followed for measurement of GSH concentration only. Shown are the measured concentrations of glutathione (GSH, in mM) in the muscles of non-healthy and healthy muscles. Individual concentration and mean values for both conditions are given. Concentration has been measured at multiple timepoints after incubation and start of absorption measurement (10 min, T10; 15 min, T15; 30 min, T30 and 60 min, T60). Statistical significance was determined with a Mann-Whitney U test. No timepoint comparison between the two conditions showed statistically significant differences.

Additional Table 2. Glutathione concentrations (GSH, mM) of the lung in non-healthy and healthy harbour porpoises. Measured values are shown for the four timepoints (10, 15, 30 and 60 mins) for individuals and mean of conditions

	Individual	T10	T15	T30	T60
non-healthy	Pph_nh2	23.270	23.953	21.798	15.632
	Pph_nh4	17.005	18.037	18.036	14.969
	Pph_nh5	25.518	27.244	26.271	19.485
	Pph_nh6	16.193	17.175	16.879	12.909
healthy	Pph_h3	14.543	15.506	15.907	12.763
	Pph_h4	35.340	35.183	31.532	21.683
	Pph_h5	24.010	23.460	21.766	16.156
	Pph_h6	11.828	12.529	11.935	9.175
mean	non-healthy	20.496	21.602	20.746	15.749
	healthy	21.430	21.669	20.285	14.944

Additional Table 3. Glutathione concentrations (GSH, mM) of the muscle of non-healthy and healthy harbour porpoises. Measured values are shown for the four timepoints (10, 15, 30 and 60 mins) for individuals and mean of conditions.

	Individual	T10	T15	T30	T60
non-healthy	Pph_nh2	6.682	5.739	4.788	3.709
	Pph_nh4	7.792	6.447	4.911	3.439
	Pph_nh5	9.495	8.109	6.079	4.285
	Pph_nh6	10.500	9.127	6.942	4.907
healthy	Pph_h3	8.063	6.643	4.819	3.113
	Pph_h4	11.961	10.067	7.435	5.170
	Pph_h5	2.482	2.384	2.352	2.329
	Pph_h6	3.844	3.446	2.873	2.168
mean	non-healthy	8.617	7.355	5.680	4.085
	healthy	6.588	5.635	4.370	3.195

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Babacığım, nihayet o an geldi ve “gerçek” bir işe gireceğim. Sabrın sonu selamettir. Umarım benimle gurur duyarsın!

13 Declaration on oath (Eidesstattliche Versicherung)

Hiermit versichere ich an Eides statt, die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen benutzt zu haben.

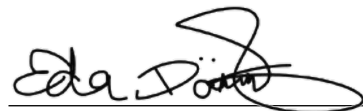
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