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Study of the long-finned pilot whale (*Globicephala melas*) bile content - An indicator of ocean health

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ABSTRACT

Globicephala melas has been harvested in the Faroe Islands for centuries. Given the distances travelled by this species, tissue/body fluid samples represent unique matrices to be considered as an integration of environmental condition and pollution status of their prey. For the first time, bile samples were analysed for presence of polycyclic aromatic hydrocarbon (PAH) metabolites and protein content. Concentrations of 2- and 3-ring PAH metabolites ranged from 11 to 25 μg mL^{-1} pyrene fluorescence equivalents. In total, 658 proteins were identified and 61,5 % were common amongst all individuals. Identified proteins were integrated into in silico software and determined that the top predicted disease and functions were neurological diseases, inflammation, and immunological disorders. The metabolism of reactive oxygen species (ROS) was predicted to be dysregulated, which can have consequences to both the protection against ROS produced during dives and contaminant exposures. The obtained data is valuable for understanding metabolism and physiology of *G. melas*.

1. Introduction

Long-finned pilot whales (*Globicephala melas*) are widely distributed in the North Atlantic Ocean, in the Mediterranean Sea, and around Antarctica and Australia (Pinzone et al., 2015; Pike et al., 2019). However, there is no known contact between the populations in the northern and southern hemisphere. Within each of their ranges, groups of individuals, called pods, move over large distances, often >100 km 24 h^{-1} (Bloch et al., 2003), and with little or no seasonal migration pattern. Given the distances travelled, samples from tissues or body fluids represent unique matrices that could be considered as an integration of the environmental condition of the large ocean regions and the pollution status of their prey. The long-finned pilot whale is a top predator, feeding on squid and a variety of fish. However, despite the constant effort to reduce anthropogenic contamination, several chemical pollutants persist in the marine ecosystem. As a high-level predator, pilot whales tend to accumulate such substances, which may affect their normal physiology and health.

Previous studies have shown the presence of environmental contaminants in its tissues, such as polychlorinated biphenyls (PCBs), brominated flame retardants and organochlorine pesticides in lipid-rich tissues, and metals and metalloids predominately in liver and kidney tissue, and also in muscle tissue (Borrell et al., 1995; Bjurlid et al., 2018; Dam and Bloch, 2000; Mendez-Fernandez et al., 2014; Gajdosechova et al., 2016). The species has been harvested in the Faroe Islands since the Norse settlement in the 9th century, with unbroken hunting records existing from 1709 to present (Zachariassen, 1993). Their meat and blubber are part of the traditional diet in the Faroe Islands (Joensen, 2009). An international research program was carried out in the 1980s to study the ecology and status of this species (Desportes et al., 1992), however, many aspects of its metabolism and physiology are still unknown.

The bile is a biological fluid commonly used as an indicator of environmental contaminations in various marine organisms (Beyer et al., 2010; Sette et al., 2013; da Silva et al., 2023). As it is the major excretion route for biotransformed (hydroxylated and/or conjugated) polycyclic aromatic hydrocarbon (PAH) metabolites, the concentration of biliary PAH metabolites can be used as a marker for assessing the ongoing (or recent) PAH exposure. In the aquatic environment, fish bile samples are often used to assess the exposure to PAHs through the

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evaluation of their metabolites (Beyer et al., 2010). The fixed wavelength fluorescence (FWF) method is used as a screening approach, either by itself or in combination with gas chromatography–mass spectrometry analysis (Aas et al., 2000; Beyer et al., 2010; Pampanin et al., 2016a, 2016b). The FWF analysis is a qualitative method, which allows semi-quantification of PAH metabolites (Beyer et al., 2010; Pampanin et al., 2016a). PAHs are found in the marine environment due to natural oil seeps, release of produced water from oil and gas platform production activities, and accidental oil spills (Pampanin and Sydnes, 2013; Pampanin and Schlenk, 2020). There are well known environmental contaminants for their carcinogenic potential, as well as their ability to cause adverse effects, such as growth reduction, DNA damage, cytotoxicity, endocrine disruption, immunosuppression, on aquatic organisms at all life stages (Pampanin and Sydnes, 2013; Pampanin and Schlenk, 2019).

The objectives of this study are to better understand the metabolism of PAHs in adult and subadult long-finned pilot whales and determine if these contaminants can influence the protein content within the bile. To the best of our knowledge, this is the first study which is using the long-finned pilot whale bile to evaluate the presence of PAH metabolites and to report the proteome content, aiming to reveal the potential of this matrix for environmental monitoring.

2. Materials and methods

2.1. Chemicals

Methanol (cat. N. 83638), acetonitrile (ACN) (cat. N. 83640), formic acid (FA) (cat. N. 20318), urea (cat. N. 28874), and dithiothreitol (DTT) (cat. N. 44149) were purchased from VWR International AS; ammonium bicarbonate (Ambic-buffer) (cat. N. 11213) and trypsin (cat. N. T1426) were acquired from Merck Life Science AS/Sigma Aldrich Norway AS.

2.2. Whale bile collection

Bile samples were obtained from individuals captured in the Faroe Islands during traditional hunting practices in 2015 and 2018 (Table 1). They were collected on-site using a syringe about 4 h after the whale were dead, transferred into 4 mL cryovials, and transported to the Faroese Environment Agency laboratory on ice, where they were kept at −80 °C prior to analyses.

Seven long-finned pilot whales were included in this study. Body length measurements were used to distinguish between adults and subadults, as previously conducted (Bloch et al., 1993). A female long-finned pilot whale was considered adult when the body length was ≥375 cm, and a male was considered adult when the body length was ≥494 cm. There were 3 adults (2 female and 1 male) and 4 subadults (3 female and 1 male) (Table 1). Long-finned pilot whales are commonly found in maternally based pods of up to 100–200 individuals (Bloch, 1998; Zachariassen, 1993), which might explain the prevalence of female individuals in this collection, although the largest individuals were avoided due to the necessity of heavy lifting to extract the samples, which required the assistance of multiple operators.

2.3. Bile sample preparation

Being the first time the FWF analysis was carried out in bile from this species, different sample preparations were tested for each individual sample: a) bile, b) bile centrifuged at 5000 ×*g* at 4 °C for 5 min, c) bile spiked with 1-hydroxypyrene (51 mg L^{-1}), and d) bile centrifuged and spiked as described in work-up c. All samples were then diluted 1:1600 with methanol/water 1:1 (v/v) and subjected to analysis.

2.4. Fixed wavelength fluorescence analysis

Samples were analysed using a Hitachi F-7000 spectrophotometer, with a slit width of 2.5 nm for both emission and excitation and a fixed voltage of 7000. A scan speed of 1200 nm/min was used and an excitation scan-range of 310 nm to 550 nm. Values were recorded at the following excitation:emission pairs: FF 290:335, FF 341:383, FF 380:430, according to Aas et al. (1998). The FF 290:335 pair indicates naphthalene-type metabolites (2- and 3-ring PAHs). Pyrene-derived metabolites (4-ring PAHs) are detected by FF 341:383 pair reading, and benzo[*a*]pyrene-type metabolites (5- and 6-ring PAHs) are detected by the FF 380:430 pair reading. As previous studies have demonstrated, the emission peak can vary (Pampanin et al., 2016a). Therefore, full scans of the emission spectra were recorded.

2.5. Bile proteome analysis

2.5.1. Sample preparation

Samples were prepared according to Wiśniewski et al. (2009) and Hernandez-Valladares et al. (2016), using a filter-aided sample preparation (FASP) for mass spectrometry–based proteomics analysis, which combines the advantages of in-gel and in-solution digestion. All proteins within the proteome are completely solubilized by urea on a standard filtration device. The peptides eluted after digestion on the filter are pure. The eluting peptides can then be analysed by mass spectrometry with no further processing aside from desalting. This allows single-run analyses of a wide coverage of the proteome. In the filter unit, proteins are alkylated by the urea buffer and are then exchanged into ammonium bicarbonate (Ambic) buffer for enzymatic digestion. The FASP-based pipeline results in a safe procedure for a deep and reproducible analysis of the proteome (Hernandez-Valladares et al., 2016). Protein concentration was measured by Pierce BCA protein assay (Thermo Fisher Scientific, Rockford, IL, USA).

2.5.2. LC-MS/MS analysis

About 0.5 μg of protein, representative as tryptic peptides dissolved in 2 % ACN and 0.5 % FA, were injected into an Ultimate 3000 RSLC system (Thermo Scientific, Sunnyvale, California, USA) connected online to a Orbitrap Eclipse mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with an EASY-spray nano-electrospray ion source (Thermo Scientific). The sample was loaded and desalted on a pre-column (Acclaim PepMap 100, 2 cm × 75 μm ID nanoViper column, packed with 3 μm C18 beads) at a flow rate of 5 μL min^{-1} for 5 min with 0.1 % trifluoroacetic acid.

Peptides were separated during a biphasic ACN gradient from two nanoflow UPLC pumps (flow rate of 250 nL min^{-1}) on a 25 cm analytical

Table 1
Details regarding the long-finned pilot whale individuals included in this study. Reported IDs are the same as used by the Faroese Environment Agency for these individuals. SubA = subadult; A = adult; F = female; M = male.

Sample code	SubA-F1	SubA-F2	SubAF3	SubA-M	A-F1	A-F2	A-M
ID	290615-6	300718-7	300718-10	300718-13	300718-3	300718-5	300718-4
Capture site	Hvannasund	Sandagerði	Sandagerði	Sandagerði	Sandagerði	Sandagerði	Sandagerði
Date	29.06.2015	30.07.2018	30.07.2018	30.07.2018	30.07.2018	30.07.2018	30.07.2018
Length (cm)	345	253	311	456	410	388	555
Maturity	subadult	subadult	subadult	subadult	adult	adult	adult
Sex	female	female	female	male	female	female	male

column (PepMap RSLC, 25 cm × 75 μm ID. EASY-spray column, packed with 2 μm C18 beads). Solvent A and B were 0.1 % FA (vol/vol) in water and 100 % ACN, respectively. The gradient composition was 5 % B during trapping (5 min) followed by 5–7 % B over 1 min, 7–22 % B for the next 74 min, 22–30 % B over 10 min, and 30–80 % B over 2 min. Elution of very hydrophobic peptides and the conditioning of the column were performed for a 10 min isocratic elution with 80 % B and 15 min isocratic conditioning with 5 % B, respectively. Instrument control was through Thermo Scientific SII for Xcalibur 1.6.

The high field asymmetric waveform ion mobility spectrometry (FAIMS) Pro interface performs gas-phase fractionation, enabling preferred accumulation of multiply charged ions to maximize the efficiency of data-dependent acquisition (DDA) routines and increase proteome coverage. Short-ion residence time in the FAIMS Pro interface electrode assembly enables the use of multiple compensation voltage (CV) settings in a single run to increase proteome coverage.

Peptides eluted from the column were detected in the Orbitrap Eclipse Mass Spectrometer with FAIMS enabled using two CVs, −45 V and −65 V, respectively. During each CV, the mass spectrometer was operated in the DDA-mode to automatically switch between one full scan MS and MS/MS acquisition. Instrument control was through Orbitrap Eclipse Tune 3.5 and Xcalibur 4.5. The cycle time was maintained at 1.5 s/CV. MS spectra were acquired in the scan range 375–1500 *m/z* with resolution R = 120,000 at *m/z* 200, automatic gain control (AGC) target of 4e5, and a maximum injection time (IT) set to Auto. The most intense eluting peptides with charge states from 2 to 6 were sequentially isolated to a AGC target value of 5e4 and a maximum IT of 75 ms in the C-trap, with the isolation width maintained at 1.6 *m/z* (quadrupole isolation), before fragmentation in the HCD (Higher-Energy Collision Dissociation). Fragmentation was performed with a normalized collision energy (NCE) of 30 %, and fragments were detected in the Orbitrap at a resolution of 30,000 at *m/z* 200, with the first mass fixed at *m/z* 110. One MS/MS spectrum of a precursor mass was allowed before dynamic exclusion for 30 s with "exclude isotopes" on. Lock-mass internal calibration was not enabled.

The spray and ion-source parameters were as follows: Ion spray voltage = 2000 V, no sheath and auxiliary gas flow, and capillary temperature = 275 °C.

2.5.3. Bioinformatics

The raw data files from the LC-MS/MS analysis were analysed using Proteome Discoverer. PD 2.5 (Thermo Fisher Sceientific Inc.) utilizing the Sequest search algorithm against the Odontoceti_tooth-whales database (downloaded from NCBI on the 6th September 2021, containing 223,633 sequences). Trypsin was set as the digestion enzyme allowing for two missed cleavages. The precursor- and fragment mass tolerance was set at 10 ppm and 0.5 Da, respectively.

Graphical analyses were performed using the R software environment (version 4.0.3: RStudio Team, 2021) using ggplot2 (version 3.3.3) for data visualization (Wickham, 2011).

2.6. Statistical analysis

For the FWF method, all samples were analysed in 5 replicates. The concentration of PAH metabolites in bile samples was expressed as mg pyrene fluorescence equivalents (PFE) mL^{-1} bile and values are reported as mean ± s.e.. Statistical comparisons were carried out using the *t*-test and $p < 0,05$. For the proteomics, data was normalized, contaminants deleted, and the normal distribution was checked. Fold changes were calculated and statistically compared by two-tailed t-test ($p < 0,05$), with significant changes in the relative abundance of proteins represented between log2(fold-change) < −1 and log2 (fold-change) > 1. Excel, Strawberry Perl, and Gnuplot programs were utilised.

2.7. Ethics

Pilot whale hunting is a long-standing tradition in the Faroe Islands (e.g., Zachariassen, 1993). The meat and blubber are used for human consumption, and therefore, for the last 30 years, the Faroese authorities established environmental monitoring using samples from the animals. The meat and blubber are non-commercially distributed in the Faroese society according to traditional rules. All animals were caught and killed according to the rules determined by the Faroese authorities. No animal was killed for the purpose of this investigation.

3. Results and discussion

The use of bile as a potential matrix in whales for determining contaminant metabolism and health assessment has not been well characterized, with present investigations limited to bile acids and similar molecules (Haslewood and Wootton, 1948; Hagey et al., 1993). In the present study, we further contributed to the investigations of whale bile to include excreted metabolites of PAHs and the whale bile proteome in a limited dataset consisting of seven individual long-finned pilot whales (one captured in 2015 and six in 2018). Although the long-finned pilot whale has been quite extensively studied in regard to pollutants such as Hg, Cd, PCBs, and perfluorinated compounds (PFAS) (e.g. Bjurlid et al., 2018; Caurant et al., 1996: Dam and Bloch, 2000), PAHs have only previously been investigated once in pilot whale blubber, where they were found in relatively low levels (Larsen and Dam, 2003). Hydroxyl metabolites of PAHs can function as biomarkers in various species based on their ability to indicate the received dose of certain compounds (Jacob and Seidel, 2002; Kim et al., 2013). In fish, metabolites are used in environmental monitoring to evaluate aquatic exposures to PAHs (Beyer et al., 2010; Pampanin and Sydnes, 2013) and are commonly linked to external exposure, organism absorption, biotransformation, and excretion, which represents an integrated response in time of the organisms' metabolism (Kim et al., 2013).

Many species are capable of metabolizing and excreting PAHs following exposure, although the exposure to low levels of PAHs has not been considered to be a major threat to aquatic health. There is currently limited information on the exposure of PAHs in higher trophic levels, such as marine mammals (Kannan and Perrotta, 2008). However, the occurrence of PAHs in marine mammals has been reported (Stimmelmayr et al., 2018) and suggested that bile can be used to validate the recent exposure to PAHs through uptake.

FWF analysis is a simple and rapid screening method to indicate the presence of classes of PAHs (2- to 6-ring compounds), and a technique that has been extensively used for the detection of PAHs in other marine species, particularly fish (Beyer et al., 2010). As bile is a biological matrix with a considerable number of interfering endogenous compounds, several modifications of sample preparation for PAH analyses by FWF were used to investigate both intra- and interindividual variations. This was also done to obtain results that could be more easily compared with previous studies performed on other marine species (Pampanin et al., 2016a, 2016b, 2019).

The centrifugation step during sample extraction that was conducted as a means to improve the optical density for PAH metabolites was not considered to be particularly useful when determining the presence of 4-, 5- and 6-ring PAH metabolites (Table S1). Subsequently, it negatively affected the results for 2- and 3-ring PAH metabolites in 3 out of the 7 bile samples. There was a great deal of variability observed between the optical density in samples analysed using the centrifugation method for PAH metabolite identification, with both increases and decreases in optical density. As there were no clear phase separations observed after centrifugation, it is hypothesised that the variability in bile sample viscosity produced a partial removal of soluble compounds when the upper layer of the sample was collected, which may have influenced the analysis.

To validate the FWF method in this newly examined matrix in

whales, bile samples were spiked with 1-hydroxypyrene, as pyrene was also used as a reference for the semi-quantification (i.e. PFE). The spiked samples resulted in a significantly higher peak in the excitation:emission pairs: FF 341:383 and FF 380:430, which confirms that FWF could detect PAH metabolites in the long-finned pilot whale bile. As previously shown, the 1-hydroxypyrene emission spectrum has one main emission band at 387 nm and two smaller bands around 425 nm, which explains the higher levels recorded in samples spiked with 1-hydroxypyrene compared with the non-spiked samples (Christensen et al., 2009; Calimag-Williams et al., 2014).

Regarding the naphthalene-type metabolites (including 3-ring PAH metabolites), values ranged between 11 and 25 PFE μg mL^{-1} of bile (mean value of 20.03 ± 2.38 PFE μg mL^{-1} of bile) and higher values were recorded in the bile of subadult individuals (23.35 ± 3.26 PFE μg mL^{-1} of bile) (Fig. 1). These levels were comparable to those reported for fish species collected in the North Sea that were potentially exposed to produced water discharges (Hylland et al., 2008; Brooks et al., 2013, 2014; Pampanin et al., 2019), and lower than values reported for Atlantic cod exposed to single PAHs, naphthalene and chrysene, and their corresponding diol metabolites (i.e. (1*R*,2*R*)-1,2-dihydronaphthalene-1,2-diol and (1*R*,2*R*)-1,2-dihydrochrysene-1,2-diol) (Pampanin et al., 2016a). However, there was not a significant difference in PFEs between subadults and adults, which is most likely due to the limited number of individuals available for this study. Although higher concentrations of PAH metabolites were recorded in the subadult individuals, differences in metabolic rates may be a contributing factor, reflecting exposure to other CYP1A inducers. Hoydal et al. (2018) previously reported that juveniles of this species have a higher ethoxyresorufin-O-deethylase (EROD) activity and *cyp1a* mRNA gene expression than adults when exposed to organohalogen contaminants (OHCs).

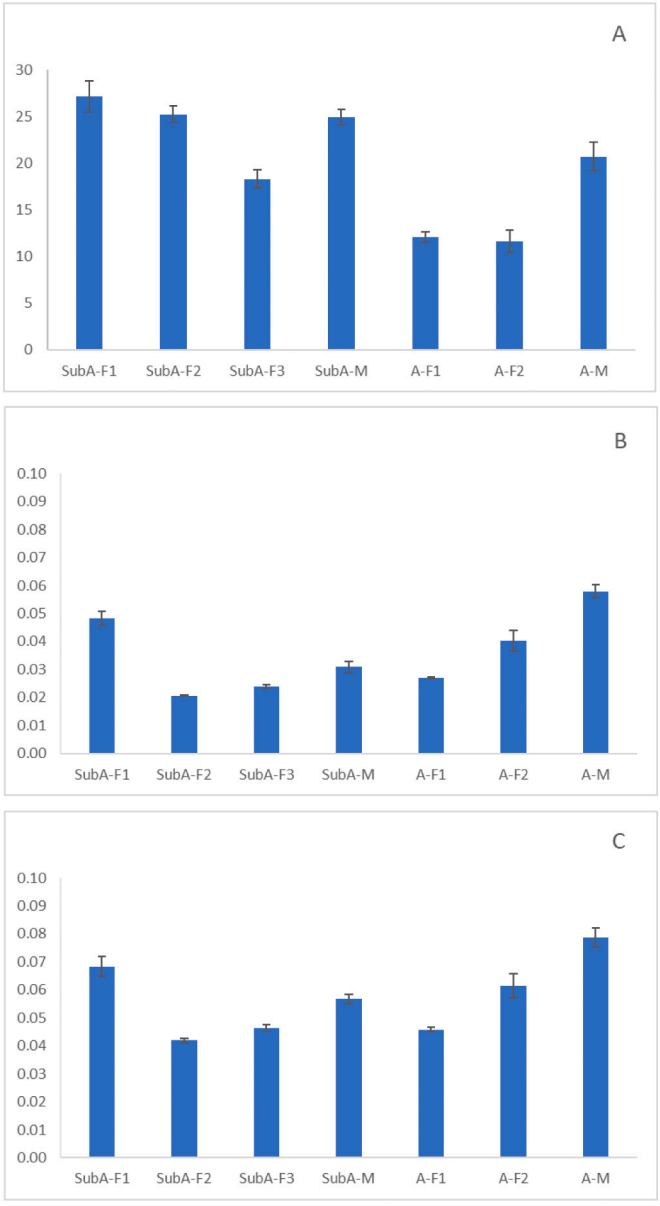

Fig. 1. Polycyclic aromatic hydrocarbon (PAH) metabolites expressed as pyrene fluorescence equivalents (PFE) μg mL^{-1} of bile., mean ± standard error (n = 5). A) 2- and 3-ring PAHs; B) 4-ring PAHs; and C) 5- and 6-ring PAHs. Single individual whale sample results are reported in the x-axis, Sub = subadult, A = adult, F = female, M = male.

The presence of PAH metabolites in *G. melas* is consistent with previous studies where biotransformation enzymes from phase I and II metabolism were correlated with contaminant exposure (White et al., 2000; Hoydal et al., 2018). In particular, the induction of *cyp1a* mRNA gene expression has been observed following PCB exposure (Hoydal et al., 2018). CYP1A is often used as a biomarker for exposure to PAHs, as it is induced by contaminants with planar configurations by binding to the aryl hydrocarbon receptor (Stegeman and Hahn, 1994). Although hypothetical, it cannot be excluded that PAH contamination could also contribute to the reported induction of cyp1a (Hoydal et al., 2018). The exposure to 2- and 3-ring PAHs might be from North Sea oil (Aas et al., 2000) or other light oil sources. Although the 2- and 3-ring PAHs are more volatile than the heavier PAHs, they are also more water soluble, and they can be available in the water column for marine organisms.

Pyrene-derived and benzo[*a*]pyrene-type metabolite mean values were very low (0.03 ± 0.01 and 0.04 ± 0.01 PFE μg mL^{-1} of bile) in subadult and adult individuals, respectively. This suggests that higher molecular weight PAHs, viz 4-rings and larger ring systems, may not be present within the diet or within the environment of sampled individuals. Considering that squid and fish represent major food sources for this species, it is likely that lower molecular weight PAHs are more representative in lower trophic level prey items, opposed to larger compounds, as reported for other contaminants (Caurant and Amiard-Triquet, 1995). The identified metabolites in bile samples confirms the exposure and metabolism of PAHs in the long-finned pilot whale and warrants additional investigation into the source of this contamination, although the potential for bioaccumulation is less than for PCBs, pesticides, and metals (Borrell et al., 1995; Caurant et al., 1996). For the quantification of single PAH metabolites, other methods can be used, for example, gas chromatography–mass spectrometry or tandem mass spectrometry (da Silva et al., 2023). As these methods are more time consuming and costly than a screening technique like FWF, they could be used as a second tiered approach, when a signal is identified.

The exposure to PAHs can depend on many factors, including sex and age. Although concentrations of certain environmental pollutants may increase with age due to bioaccumulation, females are known to transfer lipophilic persistent organochlorine compounds to offspring, which may generate an overall decline in adult females (Tanabe et al., 1987; Aguilar and Borrell, 1988; Borrell et al., 1995). Although the present study includes few individuals, the measured values of PAH metabolites are consistent with such mechanisms.

The present findings regarding PAH exposure recall the attention to the importance of studying the long-term mammalian exposure to environmental pollutants in our oceans, as also emphasised by Gajdosechova et al. (2016), while discovering high concentrations of Cd and Hg in this species. *G. melas* could be used for monitoring the quality of our oceans as demonstrated in the literature, and not only be evaluated for the risk to humans through food consumption. As higher levels of contaminants have been reported in individuals of long-finned pilot whales collected in the Mediterranean Sea (Pinzone et al., 2015), bile samples from this area could support the present finding and provide

additional information about PAH contamination, as well.

The power of omic approaches in revealing an organism's metabolism has been widely recognised (Se-Kwon and Venkatesan, 2016). In particular, the evaluation of the proteome, defined as the complete set of proteins expressed by an organism at a certain time, can provide valuable information about the organism's exposure in the environment. In contrast to the genome, which is characterized by its stability, the proteome actively changes in response to various factors, including the organism's developmental stage and both internal and external conditions. The analysis of the bile proteome has been conducted for a few species (Pampanin et al., 2014). In a previous study, the Atlantic cod (*Gadus morhua*) bile proteome was successfully assessed to develop new biomarkers of exposure to PAHs, in the form of expressed proteins affected by adductation. The bile proteome also seems to be partly species specific (Pampanin et al., 2016b, unpublished data).

Mass spectrometric analyses of bile are prone to diverse interferences from the matrix itself. The sample preparation method used in this study was previously successfully developed for fish bile in our research group (Pampanin et al., 2014). The present results demonstrated that it is possible to use this method for various aquatic organisms from fish to mammals.

A total of 662 proteins were identified in the long-finned pilot whales from the 2018 sampling (at least in 1 sample), and 61,5 % (407 proteins) of the proteins were common amongst all individuals (Fig. S1). Since there are no previous studies evaluating the bile proteome content in whales, it is not possible to do a direct comparison. However, the number of identified proteins is considered suitable for further analysis. Annotations of all identified proteins are provided in Table S2. Significantly dysregulated proteins in adult long-finned pilot whale bile compared to subadults are reported in Table 2 and Fig. S2.

Previous studies in other mammals have been focused on specific proteins from the bile or the bile conduct (e.g. Tan et al., 2017), to improve the knowledge regarding bile products, or bile metabolites, using a metabolomics approach (e.g. Ellinger-Ziegelbauer et al., 2011). Although this is not within the aim of the present study, the developed method for analysing the bile proteome can be used for such purposes in the future, allowing the identification of multiple proteins at the same time.

Long-finned pilot whale pods are reasonably stable, and usually juveniles and subadults stay with their maternal pod (Amos et al., 1993). Thus, most likely, subadults and adults are undergoing the same environmental exposures during extended periods of time. A comparison between adult and subadult proteomes may therefore point towards parameters that are influenced by age and sex-specific factors, like lactation (both on donor and recipient sides) and amounts of PAHs and their metabolism. Identified proteins were used for a comparative analysis with the Ingenuity Pathway Analysis (IPA) in silico software (QIAGEN) to identify the top predicted canonical pathways, diseases and functions, and networks.

Table 2
Significantly dysregulated proteins in adult long-finned pilot whale bile compared to subadults.

Protein identity	Fold change	p-Value
Apolipoprotein A1 (APOA1)	1.06	0.0039
Transmembrane p24 trafficking protein 10 (TMED10)	1.30	0.0062
Acyl-CoA dehydrogenase family member 9 (ACAD9)	1.07	0.0114
Transcription elongation factor A1 (TCEA1)	0.97	0.0115
SAP domain containing ribonucleoprotein (SARNP)	0.96	0.0125
Adaptor related protein complex 1 subunit mu 2 (AP1M2)	0.87	0.0142
Glutathione-disulfide reductase (GSR)	1.23	0.0199
Keratin 5 (KRT5)	0.90	0.0210
Thioredoxin domain containing 5 (TXNDC5)	1.24	0.0220
Tropomyosin 1 (TPM1)	0.97	0.0317
Keratin 6B (KRT6B)	1.12	0.0320
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma (YWHAG)	1.15	0.0365
Actin related protein 2 (ACTR2)	1.07	0.0371
Enolase 1 (ENO1)	1.20	0.0374
Y-box binding protein 1 (YBX1)	0.98	0.0424
Thymosin beta 4 X-linked (TMSB10/TMSB4X)	1.04	0.0439
Aconitase 1 (ACO1)	0.99	0.0481
AHNAK nucleoprotein (AHNAK)	1.12	0.0494
Heat shock protein family E (Hsp10) member 1 (HSPE1)	0.83	0.0498

By comparing the top predicted annotations between adult and subadult populations, it is possible to evaluate the health conditions of the long-finned pilot whales. The top predicted diseases and disorders were involved in infectious and neurological disease, immunological disease, and skeletal and muscular disorders related to neuromuscular disease (Table S3). The top predicted physiological system development and function pathways were involved in organismal survival, tissue development, hematological system development and function, organismal development, and immune cell trafficking (Table S4). Immunological disease, inflammatory disease, and organismal injury and abnormalities was amongst the top predicted networks, with a score of 54 and 29 focus proteins involved (Fig. 2). Single individuals and mass stranding events have both been reported in *G. melas* due to disease (Geraci and Aubin, 1977; Duignan et al., 1995), starvation (Geraci and Aubin, 1977), navigational error (Bernard and Reilly, 1999), or injuries (Robson, 1984), further raising concern for the health of this species in the North Atlantic.

Proteins involved in immune function have been identified in the bile of fish, and in some instances at a higher abundance relative to those found in intestinal mucus (Wu et al., 2016). Bile, containing immune molecules, can contribute to modulate intestinal immunity (Sipka and Bruckner, 2014) with bile shown to increase immune molecules upon an inflammatory response (Pavlidis et al., 2015). Therefore, the presence of immune proteins in the long-finned pilot whale's bile proteome may represent an indication of inflammation and thus potentially compromised health conditions of the whales.

Cell death and survival, post-translational modification, cellular movement, protein folding, and free radical scavenging related to the metabolism of reactive oxygen species (ROS) were the top five predicted molecular and cellular functions (Table S5). The protection against ROS is quite common in cetaceans as they face enormous challenges posed by a lack of oxygen during dives, so-called asphyxia (the integration of hypoxia, hypercapnia, and acidosis). The oxidative damage is normally limited due to an intrinsic protection against ROS by scavenging enzymes and nonenzymatic antioxidants (Tian et al., 2019). However, the metabolism of ROS is also often triggered by contaminant exposures, including PAHs. Although not within the scope of the current study, comparing the incidence of ROS relative to total contamination levels within bile could be an informative biomarker of effect in the long-finned pilot whale.

LXR/RXR activation, EIF2 signaling, NRF2-mediated oxidative stress response, acute phase response signaling, and immunogenic cell death signaling pathway were within the top 20 predicted canonical pathways (Table S4). LXR/RXR activation decreased the esterification and secretion of cholesterol esters derived from plasma membranes and is linked to cholesterol metabolism (Schultz et al., 2023). The mechanism of LXR/RXR is also connected to protection from apoptosis (Valledor et al., 2004).

Eukaryotic initiation factor 2 (EIF2) signaling is a central mechanism regulating translation initiation in response to environmental stress and regulates proinflammatory cytokine expression and is often related to bacterial infections (Wek, 2018). Under endoplasmic reticulum (ER) stress, which results in an accumulation of unfolded proteins in the ER lumen, three branches of stress sensor proteins (PERK, IRE1, and ATF6) are activated to reduce the ER load of the unfolded proteins (Ron and Walter, 2007). These transducers also activate ER stress-related molecules, such as eIF2α (Isomura et al., 2013). The presence of multiple molecules (n = 19) related to EIF2 signaling pathways suggests that there is an underlining stress condition in the individuals collected for

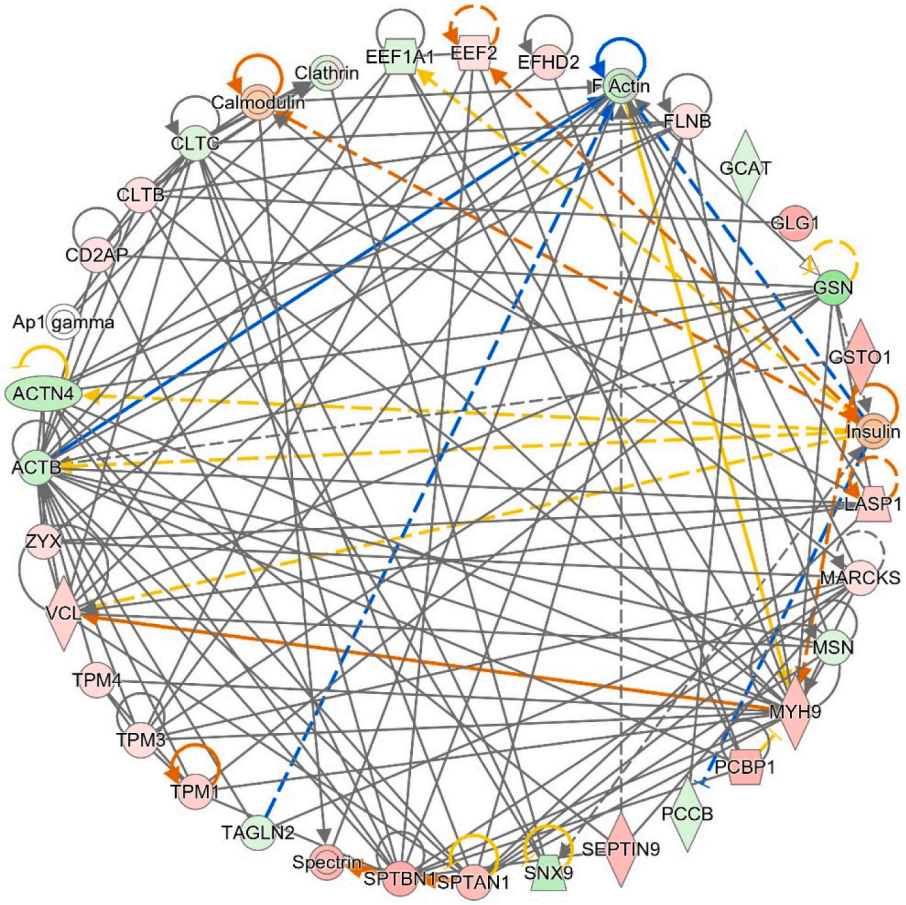

Fig. 2. Top network, predicted using the Ingenuity Pathway Analysis (IPA) in silico software, involved with Immunological disease, inflammatory disease, and organismal injury and abnormalities between adult and subadult whale bile.

this study. It is not possible to exclude that a part of this stress signal is a response to the stress the animals experience during the drive of the pod towards the beach during hunting practices.

Acute response and immunogenic cell death signaling pathways were also identified in the bile samples, which was similarly reported in a previous study involved in fish gut-liver immunity. It has been hypothesised that the bile might provide immune molecules that are used in the mucus upon inflammation (Wu et al., 2016).

The obtained results suggest that the assessment of the bile proteome can provide essential information for gut and liver immunity at the protein level, and help in understanding health conditions of *G. melas*.

4. Conclusions

This is the first known study that reports the finding of PAH metabolites in bile of long-finned pilot whales. FWF analysis identified the presence of PAH metabolites in the bile, supporting the use of this matrix as a potential biomarker in this species for future environmental monitoring studies. The FWF analysis showed the presence of 2- and 3-ring PAH metabolites, possibly originating from light oil exposure, while there were much lower levels of PAHs with four rings or more in the bile. These differences could be due to various factors like lower amounts of larger PAHs in the environment or food, or differences in metabolization and excretion efficiencies.

The bile sample preparation for analysis of the bile proteome, using LC-MS/MS, successfully identified 658 proteins, which allowed the prediction of diseases and functions and canonical pathways, to better understand physiological and general health conditions of the studied *G. melas*. This is the first known study that sheds light on the overall metabolism of log-finned pilot whales and could be used to help understand the physiology and contaminant transformations in this whale species, which is of great importance for the North Atlantic.

Follow up research, based on the present findings, is foreseen using samples from *G. melas* as indicators of the health of the ocean.

CRediT authorship contribution statement

Daniela M. Pampanin: Conceptualization, Writing – original draft, Resources, Investigation, Supervision, Visualization, Funding acquisition. **William Bossum Arnli:** Formal analysis. **Jason T. Magnuson:** Formal analysis, Investigation, Visualization, Writing – review & editing. **Giovanna Monticelli:** Formal analysis, Investigation, Visualization. **Maria Dam:** Conceptualization, Investigation, Resources, Writing – review & editing. **Svein-Ole Mikalsen:** Conceptualization, Resources, Writing – review & editing. **Magne O. Sydnes:** Conceptualization, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2023.114795.

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