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Untargeted plasma metabolomic analysis of wild bottlenose dolphins (*Tursiops truncatus*) indicate protein degradation when in poorer health

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ABSTRACT

Cumulative exposure to sub-lethal anthropogenic stressors can affect the health and reproduction of coastal cetaceans and hence their population viability. To date, we do not have a clear understanding of the notion of health for cetaceans in an ecological context; that is, how health status affects the ability of individuals to survive and reproduce. Here, we make use of a unique health-monitoring programme of estuarine bottlenose dolphins in South Carolina and Florida to determine de novo changes in biological pathways, using untargeted plasma metabolomics, depending on the health status of individuals obtained from veterinary screening. We found that individuals that were in a poor health state had lower circulating amino acids pointing towards increased involvement of gluconeogenesis (i.e., new formation of glucose). More mechanistic work is needed to disentangle the interconnection between health and energy metabolism in cetaceans to mediate potential metabolic constraints they may face during periods of stress.

1. Introduction

Top marine predators, such as cetaceans, are exposed to a wide range of environmental stressors and hence, their conservation status can be used as an indicator of broader ecosystem health (Wells et al., 2004; Bossart, 2011; Hazen et al., 2019). Anthropogenic environmental stressors, such as shipping, tourism, naval activities, contaminant exposure, coastal urbanization, and offshore energy development, can perturb environmental nutrient levels, affecting cetacean foraging abilities and inducing long-term stress. These factors are becoming a pervasive and prevalent threat to not only cetaceans but to many other marine species (Pirotta et al., 2018) and are a key priority in cetacean conservation policy (e.g. National Academies of Sciences, 2017). Anthropogenic stressors affect biological functions, and this impacts the health of individuals. Whether this impact is propagated to the ability of an individual to survive and reproduce depends on the health of individual. When in good 'health', individuals might be able to accommodate exposure to stressors but when in a poor health, individuals will lack the resilience to cope physiologically and/or behaviourally with those stressors and their effects will impact life functions (Pirotta et al., 2018).

Blubber thickness in cetaceans is used as a proxy of health to estimate the amount of energy an individual can invest in those life functions (Víkingsson, 1995). However, blubber thickness alone provides little insight into the health of cetaceans (Kershaw et al., 2019; Derous et al., 2020). This is likely due to the fact that cetaceans underwent a secondary evolutionary adaptation approximately 53 million years ago to life in water (Thewissen et al., 2009). This event drastically changed their physiology and metabolism, with a thickened subcutaneous fat layer providing energy storage, insulation and buoyancy aid (Williams et al., 1993). It is likely that due to the multiple functions of blubber beyond energy storage that it may be protected to some degree during periods of fasting (Kershaw et al., 2017). We need to move towards novel and informative health markers in cetaceans and unravel which metabolic response is linked to changes in body condition, taking the evolutionary changes of their metabolism into account.

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Infectious and inflammatory diseases are common in bottlenose dolphins (*Tursiops truncatus*) such as lobomycosis and orogenital papillomatosis being one of the most common diagnoses in wild individuals (Reif et al., 2008b). This can have a profound effect on the energy metabolism of cetaceans, as infection may cause immunopathologic perturbations (Bossart et al., 2019). While we can obtain serological evidence of those health challenges, we do not understand the key biological functions affected by diseases and their potential consequences for the ability of individuals to maintain life functions. We can use untargeted metabolomics to investigate biological pathway responses to environmental challenges. For example, we recently used this approach to identify a "fingerprint" associated with the physiological response to fasting in managed bottlenose dolphins (Houser et al., 2021). We identified a dynamic network marker that has the potential conservation application of assessing energy state balance in at-risk wild dolphins. Here, we use untargeted plasma metabolomics to determine changes in biological pathways depending on the categorical health status of wild individuals estimated from veterinary screening from two different locations: Charleston and the Indian River Lagoon, US. However, it must be noted that this study was performed on free-ranging animals and other underlying factors such as feeding state (i.e., last time to meal) may impact their metabolic response. Bottlenose dolphins consume a wide range of prey species, such as sciaenids (i.e. drums, croakers, and sea trout) and many of the same prey species were reported for both the Charleston and the Indian River Lagoon dolphins (Barros and Odell, 1990; Barros, 1993; Pate and McFee, 2012). In general, bottlenose dolphins feed throughout the day and night, consume small proportions of their total intake in brief bounds, and seem to feed whenever is necessary (Wells et al., 2013a). Since they feed throughout the day there should not be extremes of feeding state among the dolphins sampled. However, differences in feeding state may be a confounding effect on the health assessment and the overall phenotype of the dolphins.

2. Material and methods

We estimated associations between health status and energy metabolism by analysing plasma metabolites from two wild Atlantic bottlenose dolphin (*Tursiops truncatus*) populations; i) in Charleston Harbor, South Carolina (CHS) and ii) in the Indian River Lagoon, Florida (IRL). In model species (e.g., human and rodent studies), metabolomic analysis can directly reflect the biochemical activity underlying a certain phenotype in cells or tissues and hence reflects the molecular phenotype. However, in this study we identified the metabolomic profile in free-ranging species and potential confounding factors may influence the results (e.g., feeding ecology). By using samples from two different physiological states (i.e., diseased vs normal), we expect to see variability in their metabolism. Metabolite analysis has either been done in exhaled breath in cetaceans (Aksenov et al., 2014; Zamuruyev et al., 2016), with limited metabolite identification performed on a tissue level (Misra et al., 2019).

2.1. Study sites, subjects and sample collection procedures

Samples were collected as part of the Bottlenose Dolphin Health and Risk Assessment (HERA) Project, a multi-disciplinary research project initiated as a collaborative effort between the National Ocean Service's Center for Coastal Environmental Health and Biomolecular Research, South Carolina and Harbor Branch Oceanographic Institution, Florida. This project aimed to examine the potential association between health and environmental conditions in two bottlenose dolphin populations along the eastern coast of the United States: the IRL, Florida, and the estuarine waters of CHS, South Carolina (Fiar and Bossart, 2005; Schaefer et al., 2009). Free-ranging dolphins were temporarily constrained and physically examined, blood and tissue samples were collected and cultures for microbiological investigation were obtained in CHS during 2013 ($n = 19$) and in the IRL in 2011 ($n = 26$). The protocols for the capture and release method were previously published and described in detail (Fair et al., 2006). Samples from dolphins were collected under National Marine Fisheries Permit No. 14352-03 issued to Dr. Gregory Bossart and approved by the Florida Atlantic Institutional Animal Care and Use Committee (IACUC) under Protocol #A10-18. Here, we focus our metabolomic analysis (i.e. diseased vs healthy) separately for each site to avoid potential between-site confounders, particularly as poor health profiles were different between the two locations (Reif et al., 2008a). During the health assessments, dolphins were classified by a team of veterinarians into 3 states: normal, concerned and diseased using the method described in Reif et al. (2008a). We combined the diseased and concerned cases or those with visible pathological conditions and disease markers in blood as diseased animals (Table S1). This resulted for CHS in 4 animals classified as healthy (i.e., absence of pathological conditions or disease markers in blood) and 15 as diseased (i.e., concerned plus diseased animals); and for IRL in 12 animals as healthy and 14 as diseased. Identification of the feeding state was not part of the health assessments.

To minimize variables between the two sites, sampling was restricted to summer at both sites so that water temperatures would be similar. Blood samples were collected in EDTA vacutainer tubes within the first 10 min after capture using a 19-gauge butterfly catheter. Plasma was collected from these samples and stored at −80 °C until analysis. Age was determined by examining the postnatal dentine layers of an extracted tooth (Hohn et al., 1989). The majority of the samples originated from male individuals and the average age for CHS dolphins was 17.8 years and for IRL was 14.4 years.

2.2. Stress hormone analysis

Stress hormone levels from these samples were previously published, along with health and other metrics (see Fair et al., 2017). Here, we report the concentration of key markers in blood related to stress status (i.e., levels of adrenocorticotropic hormone (ACTH) (pg/ml), cortisol (μg/dl) and aldosterone (pg/ml)). Data were checked for normality and equal variance, and where appropriate data were transformed. We then fitted a One-way ANOVA for each stress marker with health status as the explanatory variable. A significance threshold was set to a p-value < 0.05. Data was visualised in R version 3.6.1 (Ginestet, 2011; R Core Team, 2019) using ggplot2.

2.3. Metabolomic analysis

Pre-processing of the samples include deproteinizing and centrifugal evaporation. Samples were prepared according to established protocols (Cipriano et al., 2015) and were resuspended in 10 μL of 40 mg/mL Omethylhydroxylamine hydrochloride in pyridine. After shaking for 90 min at 30 °C (1400 rpm), 90 μL of *N*-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) + 1% trimethylchlorosilane (TMCS) (Thermo Scientific, Lafayette, CO) was added to the samples. After another 30 min of shaking at 37 °C (1400 rpm), samples were centrifuged (21,100*g*) for 3 min, and 50 μL of the supernatant was added to an autosampler vial. Samples were spiked with 0.25 μL of a retention time standard solution consisting of fatty acid methyl esters (FAMEs) and an internal standard of nonadecanoic acid methyl ester dissolved in dimethylformamide. Metabolite identification was performed using coupled two-dimensional gas chromatography/mass spectrometry (GCxGC-MS) with a LECO Pegasus 4D instrument (Aglient 7890A gas chromatograph and 7683B autosampler) (Cipriano et al., 2015). Complete method details can be found in the method section and supplemental information of Cipriano et al. Each experimental sample was analysed with technical duplicate injections, with sample order randomized to limit batch effects. A pooled quality control sample was created from a small aliquot of each sample.

We used the library MetaboDiff 0.9.3 in R version 4.0.2 (R Core

Team, 2019) to estimate the change in metabolite expression between normal and diseased individuals for each site separately (Mock et al., 2018). Dataset was checked for missing values (15%) but did not exceed the recommended 40% cut-off to perform k-nearest neighbor imputation (Armitage et al., 2015). Data was then normalised to ensure the distribution of measurements was comparable across the dataset data (Huber et al., 2002). A data visualization was performed using a principal component analysis (PCA) as a linear and t-distributed stochastic neighbor embedding (tSNE) as a non-linear dimension reduction technique, as well as a heatmap for the intensity values of the metabolites, including k-means clustering ($n = 4$ to represent the two treatment levels applied to both populations). Metabolomic profiles between diseased and healthy dolphins were contrasted using the diff_test function in the MetaboDiff R package, which uses the Student's *t*-test (Mock et al., 2018) and visualised using ggplot2 (Ginestet, 2011). Due to the small numbers of significantly different metabolites, we did not perform pathway enrichment analysis or overrepresentation analysis. We mapped the known metabolites to biological functions based on the description given on the Human Metabolome Database 4.0 (hmdb, https://hmdb.ca/) (Wishart et al., 2018).

3. Results

3.1. Stress hormone markers

Data on blood chemistry (previously published (Fair et al., 2017)) from wild Atlantic bottlenose dolphins were assessed to determine the difference in potential markers in the blood chemistry linked to stress based on their health classification (Fig. 1). None of the stress hormones were significantly different between diseased and normal individuals at the IRL site. Cortisol was significantly elevated in diseased individuals at the CHS site (one way ANOVA, $F_{1,16} = 6.4251$, p-value $= 0.022$).

3.2. Overall metabolomic profile at the different sites

A total of 345 peaks were determined in plasma as candidate metabolites, of which 202 were unable to be identified and were classified as unknowns. A heatmap of all metabolites (including unknowns) indicated little difference in overall metabolic signature between the two health states (Fig. 2). The K-mean clustering ($n = 4$) did not cluster the individuals according to health state but according to sites. A principal component analysis (PCA) indeed showed that PC1 explained 33% of the variation on dimension 1, showing a separating according to sites (Fig. 3). In agreement with the PCA, the first two dimensions in the tSNE plot did not reveal a distinct difference in the metabolomic profiles between diseased and healthy bottlenose dolphins (Fig. 3).

3.3. Differentially changed metabolites between healthy and diseased animals at each site

As the population of CHS and IRL dolphins exhibited differences in their poor health profiles, we analysed these two sites separately. We compared diseased versus healthy individuals at both sites. At both sites, a total of 20 metabolites were significantly different between the diseased and healthy animals (Fig. 4, Table S2). In both sites, L-proline and Indolelactic acid was reduced in plasma of diseased animals, and L-lactic acid and Pyroglutamic acid were increased in the plasma of diseased animals (Fig. S1).

Due to the low numbers of differentially known identified metabolites, we did not perform pathway enrichment analysis. However, we did map individual metabolites to major biological processes based on the Human Metabolome Database (Table 1). The majority of the metabolites were involved in amino acid metabolism.

4. Discussion

Infectious and inflammatory diseases can have a profound effect on the energy metabolism of cetaceans (Bossart et al., 2019). Feeding state could not be measured in this study and as such the results may be confounded by an underlying difference in feeding behavior. Nevertheless, in this study we were able to identify metabolites that were significantly different between two health profiles in bottlenose dolphins. Although the dolphin populations at CHS and IRL displayed different poor health profiles (Reif et al., 2008a), we did identify four

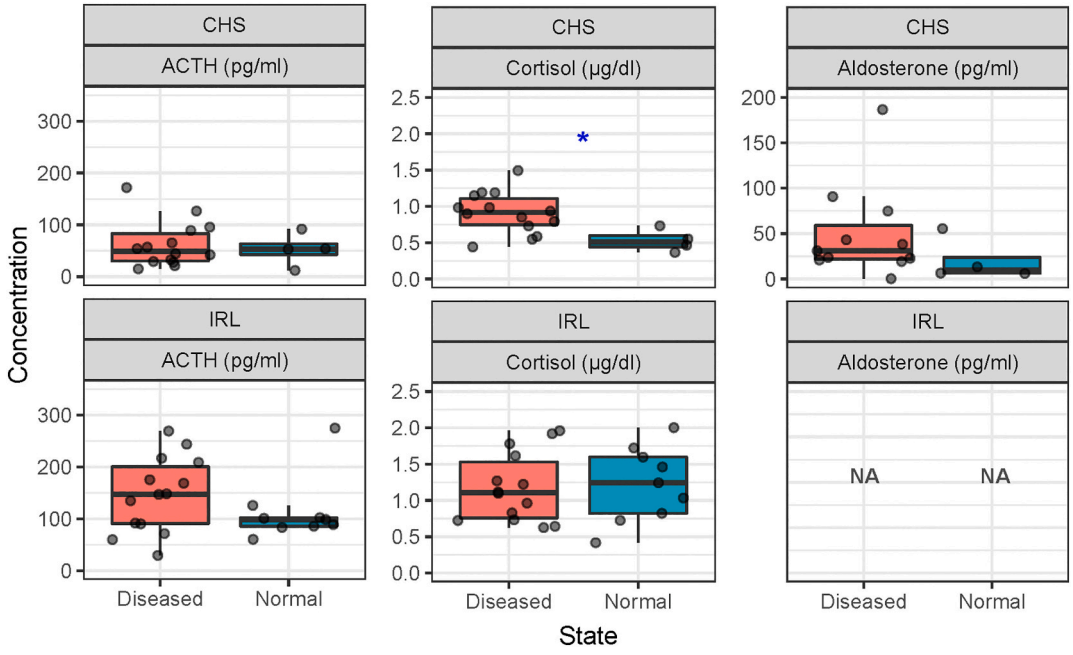

Fig. 1. Stress hormone markers identified in blood of wild Atlantic bottlenose dolphins (*Tursiops truncatus*) sampled from Charleston (CHS) and Indian River Lagoon (IRL) for two health states: normal and diseased. Stress hormones for CHS and IRL were previously published as a comparison with managed dolphin populations (Fair et al., 2017). Levels of adrenocorticotropic hormone (ACTH) are expressed as pg/ml, cortisol as µg/dl and aldosterone as pg/ml on the y-axis. Significance in hormone level between the two health states is indicated by the blue asterisk.

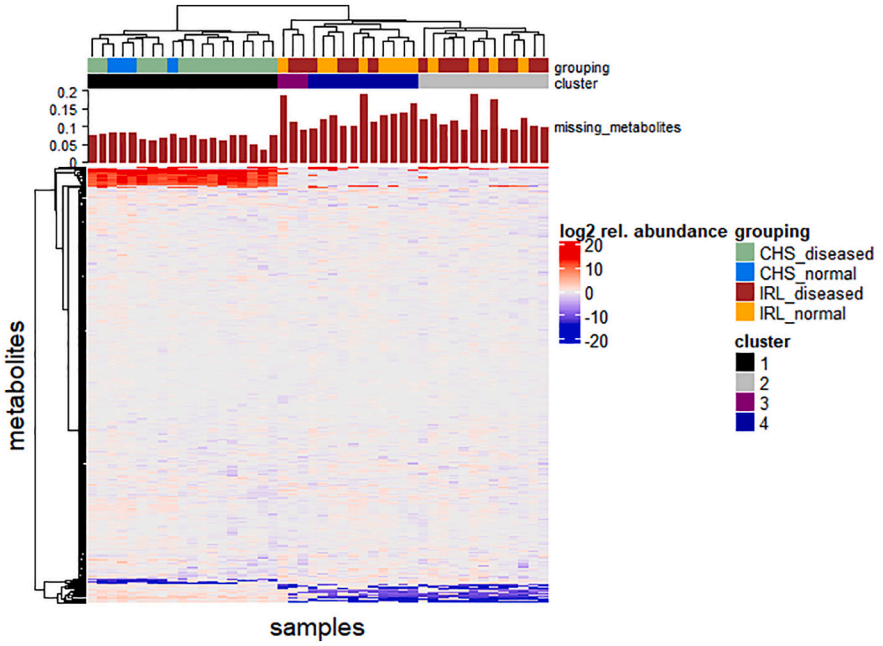

Fig. 2. A heatmap of the $\log_2$ transformed intensity values of 345 plasma metabolomics analytes in wild Atlantic bottlenose dolphins (*Tursiops truncatus*) from Charleston (CHS) and Indian River Lagoon (IRL) for two health states: normal and diseased. Each row represents one metabolite, and each column represents each sample/individual dolphin. Columns are annotated according to group with green for diseased CHS animals, blue diseased IRL animals, red normal CHS animals and yellow normal IRL animals. Intensity value of the metabolite is represented as $\log_2$ values with missing values indicated in the red bars. Missing metabolites are shown as a proportion, the grouping is based on our pre-defined groups of the health classes of the dolphins, and the cluster refers to the clustering identified by the K-mean clustering approach.

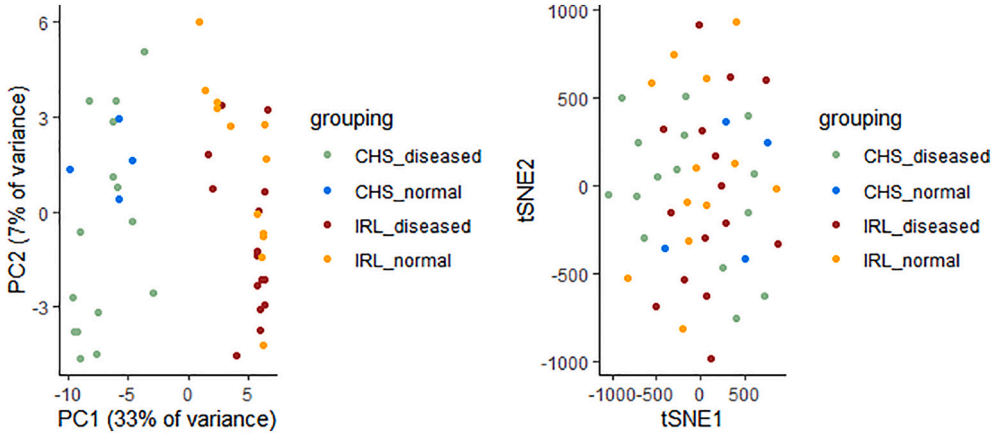

Fig. 3. A principal component analysis (PCA, left) and t-distributed stochastic neighbor embedding (tSNE, right) of 345 plasma metabolomics analytes in wild Atlantic bottlenose dolphins (*Tursiops truncatus*) from Charleston (CHS) and Indian River Lagoon (IRL) for two health states: normal and diseased. Individuals are coloured according to group with green for diseased CHS animals, blue diseased IRL animals, red normal CHS animals and yellow normal IRL animals.

metabolites of which the plasma intensity was changed in the diseased animals when compared to the healthy ones. L-Lactic acid and Pyroglutamic acid were increased in diseased dolphins and L-proline and Indolelactic acid were reduced in the diseased dolphins at both sites. Lactic acid, the conjugate base of lactate, was significantly higher in the diseased group at both sites. Lactic acid is produced during anaerobic metabolism but also plays a much larger role in cell signalling, metabolism, inflammation and shows a turnover in aerobic conditions in humans and mammalian model systems (Brooks et al., 2021). Cetaceans have evolved coping mechanisms to recover from oxygen deprivation caused by breath-holding during dives, including an increased capacity to process lactic acid (Costa, 2007; Tian et al., 2017). The populations located at IRL and CHS live in extreme shallow water and thus their dives are unlikely to exceed their aerobic dive limit for this species. As such the small increase in lactic acid observed in diseased animals is likely not due to changes in their capability to clear lactic acid levels after deep dives. Increased lactic acids is also associated with inflammatory diseases/states due to higher energy demands (Pucino et al., 2017). Activated immune cells (e.g., lymphocytes) rely mainly upon glycolysis and as a consequence lactate is produced (Pucino et al., 2017). However, lactate was also found to reduce inflammation and playing a key role in suppressing the activation of NF-κB in macrophages of mice (Hoque et al., 2014). The majority of the diseased dolphins exhibited signs of an immune or inflammatory disease, and thus the increased levels of lactate may be an inflammatory response to those diseases.

Pyroglutamic acid, intermediate substrate involved in the synthesis of glutathione, was also increased in diseased dolphins at both sites. Glutathione levels can be reduced due to oxidative stress and this can secondarily lead to an increased production of pyroglutamic acid (Palmer and Alpern, 2010). Under normal physiological conditions, mammalian metabolism produces reactive oxidative species (ROS) and these are cleared by antioxidant defence systems. Increased generation of ROS can occur during certain pathological conditions and leads to oxidative stress causing cellular damage (Yu, 1994). Glutathione is an anti-oxidant protecting the cells from oxidative stress and thus decreased concentrations of glutathione are associated with increased generation of ROS (Forman et al., 2009). Elevated levels of pyroglutamic acid in humans are often associated with patients with infections or

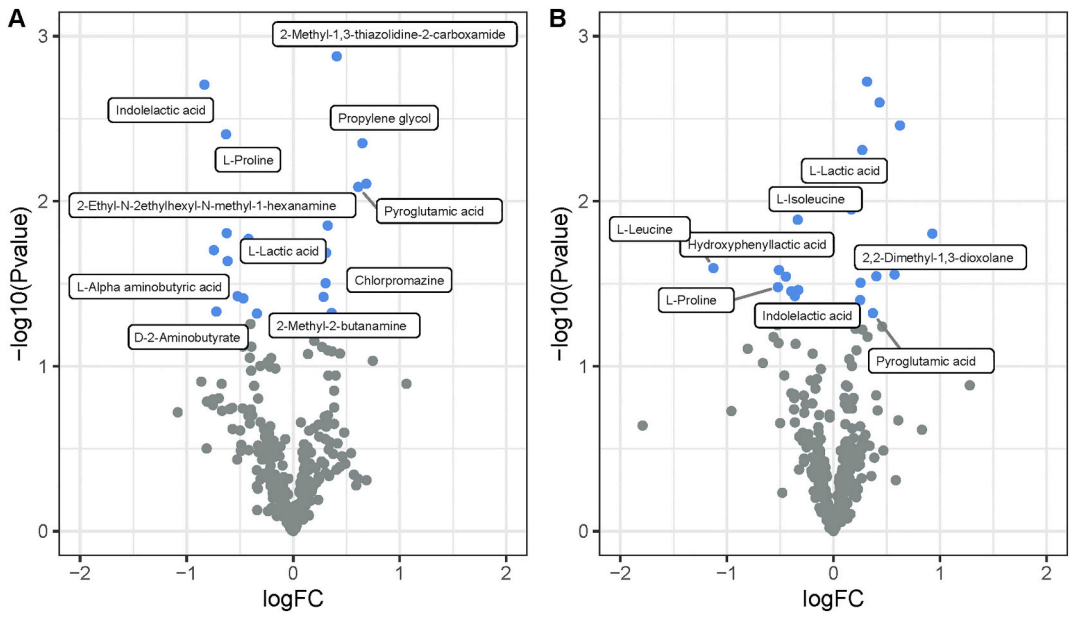

Fig. 4. Differential metabolites at the sites (A) Charleston and (B) Indian River Lagoon for diseased animals compared to healthy animals. X-axis represents the $\log_2$ transformed fold change and y-axis the $\log_{10}$ transformed raw *p*-value. Significant metabolites are coloured blue and non-significant metabolites grey. Those significant identified metabolites are labelled on the figure.

sepsis (Gueta et al., 2020). The elevated levels of pyroglutamic acids in diseased dolphins probably reflects the generation of ROS due to their immune or inflammatory response and the need to clear these by antioxidant mechanisms of glutathione. The depletion of glutathione will then lead to an increase in pyroglutamic acid levels, which was observed in these dolphins.

L-Proline, reduced in diseased dolphins, is an amino acid that plays a role in the biosynthesis of proteins, antioxidant defence mechanisms and immune responses (Van Meijl et al., 2010; Wu et al., 2011). In addition, it is a signalling molecule that serves as a cellular energy status sensor and regulates gene expression to activate biological pathways crucial in health and disease (Phang et al., 2008). The lower levels of circulating L-proline in diseased dolphins may be due to the higher amount of ROS that is generated during the inflammatory response and the need for a higher antioxidant defence system (i.e., L-proline being used up). In addition, L-proline also plays a role in providing sufficient energy during inflammatory responses via gluconeogenesis (i.e., muscle breakdown to convert to glucose). Bottlenose dolphins fasted for 24 h show a rapid switch towards using lipids and amino acids to supply the shortfall in energy (Houser et al., 2021) instead of glycogen breakdown as expected in short-term starvation in model species (Berg et al., 2002). As such, protein breakdown plays a bigger role than originally anticipated in energy metabolism of cetaceans. This could have consequences for their survival if muscles are being wasted to supply energy during periods of inflammatory or immune responses and if foraging is even further disrupted (e.g., human caused stressors or changed diving behavior). This is similar to what is observed in rodents and humans, where diseases are often associated with muscle protein loss (Tiao et al., 1994; Lecker et al., 1999). This metabolic response then provides the infected organism with a source of energy by converting amino acids into glucose (gluconeogenesis).

Indolelactic acid was lower in diseased dolphins at both sites. Indolelactic acid is a tryptophan metabolite, which is metabolized via the kynurenine pathway or via series of indoles. Similar to L-proline, Indolelactic acid may play a role in providing energy to fight infections. The kynurenine pathway metabolises tryptophan to acetyl CoA and eventually nicotinamide adenine dinucleotide (NAD+), which is essential to create energy and regulate cellular processes. In humans, metabolites from the kynurenine pathway are considered biomarkers for muscle breakdown and are used to monitor diseases such as amyotrophic lateral sclerosis (Tan and Guillemin, 2019). In addition, depletion of indole-derived metabolites in serum has been identified in both murine and human colitis (Alexeev et al., 2018), a disease characterized by colon inflammation. Several of the diseased dolphins displayed evidence of gastric inflammation. Hence, the changes we observed here in the animals with a diseased status may indeed reflect protein breakdown for gluconeogenesis to provide energy for the inflammatory response.

4.1. *Diseased dolphins at CHS show evidence of chemical exposures and protein degradation*

The CHS dolphins displayed lower levels of other amino acids (i.e., D-2-Aminobutyrate, L-Alpha-aminobutyric acid and 2-Methyl-1,3-thiazolidine-2-carboxamide) and increased levels of 2 chemical compounds related to pharmaceuticals (Chlorpromazine and Propylene glycol). D-2-Aminobutyrate and L-Alpha-aminobutyric acid are both conjugates of L-2-aminobutyric acid. The glutathione consumption during oxidative stress is accompanied by production of ophthalmic acid originating from 2-aminobutyric acid (Irino et al., 2016). Decreased levels in its conjugates (i.e., D-2-Aminobutyrate and L-Alpha-aminobutyric acid) would reflect a consumption of 2-aminocutyric acid in mechanism related to clearing ROS and oxidative stress defence mechanisms, which is in agreement with the metabolites discussed above. 2-Methyl-1,3-thiazolidine-2-carboxamide is a L-cysteine derivative and this is an essential compound for the formation of glutathione. Lower levels of 2-Methyl-1,3-thiazolidine-2-carboxamide may reflect the increase in antioxidant mechanisms.

Interestingly, the diseased dolphins located at CHS also displayed higher levels of 2 chemical compounds detected in their plasma. Propylene glycol, one of the identified compounds, is released in the aquatic environment via waste streams from the use and disposal of this compound in de-icing solutions. It is rapidly degraded in all environmental media and as such it is not expected to persist or bioaccumulate in aquatic organisms (Registry Agency for Toxic Substances and Disease, 1997). It is therefore unclear why diseased dolphins in CHS would have higher levels of propylene glycol in their plasma. The other chemical compound, Chlorpromazine, is used as a antipsychotic drug in humans. Significant levels of antipsychotics have been detected in rivers, lakes

Table 1
Biological function of significantly altered metabolites at Charleston (CHS) and Indian River Lagoon (IRL) for diseased versus healthy individuals based on the Human Metabolome Database. The numbers in the column "Increased or decreased" represents the logFold Changes of diseased individuals vs healthy individuals.

Location	Metabolite	HMDB ID	Increased or decreased	Function
Both CHS and IRL	L-Lactic acid	HMDB0000190	Increased CHS: 0.324 IRL: 0.270	Produced in the muscles, marker for energy failure
	L-Proline	HMDB00162	Decreased CHS: −0.630 IRL: −0.521	Amino acid that is used in the biosynthesis of proteins
	Indolelactic acid	HMDB0000671	Decreased CHS: −0.833 IRL: −0.330	Tryptophan metabolite, amino acid precursor for serotonin
	Pyroglutamic acid	HMDB0000267	Increased CHS: 0.610 IRL: 0370	Cyclized derivative of L-glutamic acid, formed nonenzymatically from glutamate, glutamine, and gamma-glutamylated peptides
CHS	2-Ethyl-N-(2-ethylhexyl)-*N*-methyl-1-hexanamine	NA	Decreased −0.624	NA
	2-Methyl-1,3-thiazolidine-2-carboxamide	HMDB0062599	Increased 0.409	Alpha amino acid
	2-Methyl-2-butanamine	NA	Increased 0.285	NA
	Chlorpromazine	HMDB0014620	Increased 0.305	A drug which is used for the treatment of schizophrenia, potential toxic compound
	D-2-Aminobutyrate	HMDB0000650	Decreased −0.721	Alpha amino acid
	L-Alpha-aminobutyric acid	HMDB0000452	Decreased −0.615	Alpha amino acid
	Propylene glycol	HMDB0001881	Increased 0.649	Used as a solvent for intravenous, oral, and topical pharmaceutical preparations
IRL	2,2-Dimethyl-1,3-dioxolane	NA	Increased 0.254	NA
	Hydroxyphenyllactic acid	HMDB0000755	Decreased −0.512	Tyrosine metabolite, amino acid precursor for dopamine and norepinephrine, reduced in patients with unusual gut microflora
	L-Isoleucine	HMDB0000172	Decreased −0.335	Essential amino acid, involved in stress, energy and muscle metabolism
	L-Leucine	HMDB00687	Decreased −1.127	Essential amino acid that is used in the biosynthesis of proteins

Summary			
Site	Increased	Decreased	Total
CHS	10 (3 unknowns)	10 (5 unknowns)	20 (8 unknowns)
IRL	11 (8 unknowns)	9 (4 unknowns)	20 (12 unknowns)

N/A = not available.

and sea water and concentration levels of Chlorpromazine were above the predicted no effect concentration (PNEC) threshold in hospital wastewater (Reichert et al., 2019). The environmental impact or the potential of bioaccumulation through the food web for chlorpromazine is not well known. Levels have been detected in crustaceans (Richmond et al., 2018) and experimental manipulations show a major biological impact (mortality) after a prolonged exposure time to this drug in *Thamnocephalus platyurus* (Nalecz-Jawecki and Persoone, 2006). Exposure to chlorpromazine also lead to a lower feeding rate in *Daphnia magna* compared to controls (de Alkimin et al., 2020). Juvenile African sharptooth catfish (*Clarias gariepinus*) that were allowed to recover after being exposed to different levels of chlorpromazine showed increased levels of white blood cells and decreased levels of hemoglobin, erythrocytes and packed cell volume (Okpe et al., 2021). When exposed to this drug, juvenile catfish also showed increase in oxidative stress parameters (Atama et al., 2020). Based on these ecotoxicological effects, it was concluded that the disposal of chlorpromazine should be strictly regulated to avoid impact on non-target organisms. Although little work is done on marine mammals, Chlorpromazine may have ecotoxicological impacts and thus should be considered as a novel contaminant. It is however unclear why diseased dolphins would have higher levels of this drug in their system compared to healthy animals. A possible explanation is their liver function may be impaired due to the illness and thus the diseased animals may not be able to clear this drug from their system at the same rate as healthy animals. However, this is a possible explanation, and another likely scenario might be a case of misidentification. Raw data as peaks are matched to metabolites based on retention time and mass. A common issue for metabolomics is peak misannotation (Lu et al., 2017).

4.2. Diseased dolphins at IRL show evidence of protein degradation

Three more amino acids were reduced in the diseased dolphins located at IRL: L-leucine, L-isoleucine and hydroxyphenyllactic acid. We see different amino acid profiles at the different sites, and this may be due to their different disease profile. The lower levels of these 3 amino acids again indicate that diseased dolphins rely on these amino acids to create extra energy and may exhibit muscle wasting. Lower levels of hydroxyphenyllactic acid (a precursor of amino acid tyrosine) are linked to human diseases with muscle atrophy such as Huntington's disease (Zielonka et al., 2014; Rosas et al., 2015). In addition, hydroxyphenyllactic acid may also play a role as antioxidant clearing ROS generated by inflammatory and immune response (Beloborodova et al., 2012). L-Leucine is considered a ketogenic amino acid (i.e., transformed into ketones instead of glucose) while L-isoleucine is considered to be both ketogenic and glucogenic in humans and rodents. Interestingly, during prolonged fasting (i.e. 72 h), dolphins do not seem to exhibit any signs of ketosis (Ridgway, 2013). This is surprising as a diet void of

carbohydrates induces a shift towards beta-oxidation of fatty acids in land mammals and ketone bodies are produced (Westman et al., 2007). Dolphin diet has a high fat and protein content and is almost devoid of carbohydrates (Wells et al., 2013b) and hence we would expect production of ketone bodies during starvation. However, a recent study showed that cetaceans exhibit genomic mutation in key ketogenesis genes, suggesting that they lost the ability to produce ketones (Wolfgang et al., 2021). Wolfgang et al. showed that hepatic cetacean tissue indeed does not express β-hydroxybutyrate (βHB, ketone body) in comparison to murine tissue. A 24 h fasting study with bottlenose dolphins showed indeed no signs of ketone production as is expected in fasting in model species (Berg et al., 2002; Houser et al., 2021). As such, gluconeogenesis from example amino acids, glycerol, and recycling of other 3-carbon intermediates (e.g., lactate/pyruvate) may be sufficient to produce glucose needed for those tissues that are glucose dependent (i.e., brain and red blood cells) (Champagne et al., 2006; Champagne et al., 2012a; Champagne et al., 2012b). However, very little work has been done into gluconeogenesis in cetaceans and we can only speculate that this is indeed the case.

4.3. Potential metabolic consequences of stress on diseased individuals

We do need to note that the capture-release method used here can induce stress for the animals and this should be taken into account, even though its effects are applied to individuals of both health states. Here, we found a significant increase in cortisol levels in diseased individuals at the CHS site. Stress response is regulated by the hypothalamic-pituitary-adrenal axis by releasing stress hormones. Wild dolphins have a typical mammalian response to acute stress with circulating levels of stress hormones elevated (Thomson and Geraci, 1986; St. Aubin et al., 1996; Suzuki et al., 2003; Fair et al., 2014; Champagne et al., 2018). Functional manipulation studies in model organisms show that increased stress leads to a shift towards glycogenolysis (i.e. breakdown of glycogen) and gluconeogenesis (i.e. formation of glucose from amino acids) (Sherwin and Sacca, 1984; Khani and Tayek, 2001). This withdrawal from energy stores is necessary to maintain the exercising muscles and the brain (Romero and Wingfield, 2016) allowing for a rapid response and optimises survival from immediate threats. Given that the capture/samples procedure was applied to all individuals, we can speculate that the diseased state may experience increased stress. However, it is hard to disentangle if diseased animals were less able to cope with the capture/sample procedure and thus display a potential higher stress level. Or that stress levels of these animals were higher prior to the capture/sample procedure. Nevertheless, this data may shed some light on how diseased individuals are able to cope with extra stressors.

5. Conclusions

Finding ecologically relevant markers of "health" for cetaceans is key to enabling the use of these species as sentinels of the sea as well as to understanding the cumulative impact of stressors on the conservation of cetaceans emerging from our current rapid diversification of human marine activities (e.g. National Academies of Sciences, 2017; Derous et al., 2020). It is pivotal for health to be examined in the context of how an animal will invest in reproduction and survival on a physiological level when they are exposed to sub-lethal stressors (e.g., noise, contaminants, prey limitation) caused by anthropogenic disturbances. Energy metabolism plays an important role in reproduction and survival and it is currently unclear how multiple stressors are integrated in the energy metabolism of the targeted species and how this affects health. Here, we were able to distinguish between the 2 dolphin health groups and identified different metabolomic signatures. We do need to note that the detection of molecules are dependent on the methods used here and thus molecules that not easily derivatized and volatilized will not be detected. In addition, only metabolites with known peak retention times will be present in the database used for annotation. As such, our dataset has unknown metabolites that show a significant different between the two health states. Those unknown metabolites will play an important biological role in signalling/regulating metabolism and may be involved in other pathways than the amino acid metabolism identified here. Overall, we found that in wild bottlenose dolphins, those with a challenged 'ecological health' had lower circulating levels of amino acids, suggesting conversion to glucose. We also found higher levels of L-lactic acid, which may an indicator of a biological repsonse to inflammation. Taken together, our results suggest that the disease state had metabolic consequences, and these may constrain the way cetaceans could cope with extra stressors (e.g., human disturbances) Plasma metabolite profiles in cetaceans have some stark contrasts with model species. For example, cetaceans have higher levels of urea and differ in other key metabolites (Miyaji et al., 2010). Hence, more fundamental mechanistic work is needed to disentangle the interconnection between health and energy metabolism in cetaceans to mediate potential metabolic constraints they may face. This may provide important insight on how we deal with conservation policies and on our understanding of the effect of stressors on population dynamics.

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Declaration of competing interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbd.2022.100991.

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