Biomarkers of Whale Shark Health: A Metabolomic Approach

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Abstract

In a search for biomarkers of health in whale sharks and as exploration of metabolomics as a modern tool for understanding animal physiology, the metabolite composition of serum in six whale sharks (Rhincodon typus) from an aquarium collection was explored using 1H nuclear magnetic resonance (NMR) spectroscopy and direct analysis in real time (DART) mass spectrometry (MS). Principal components analysis (PCA) of spectral data showed that individual animals could be resolved based on the metabolite composition of their serum and that two unhealthy individuals could be discriminated from the remaining healthy animals. The major difference between healthy and unhealthy individuals was the concentration of homarine, here reported for the first time in an elasmobranch, which was present at substantially lower concentrations in unhealthy whale sharks, suggesting that this metabolite may be a useful biomarker of health status in this species. The function(s) of homarine in sharks remain uncertain but it likely plays a significant role as an osmolyte. The presence of trimethylamine oxide (TMAO), another well-known protective osmolyte of elasmobranchs, at 0.1–0.3 mol L⁻¹ was also confirmed using both NMR and MS. Twenty-three additional potential biomarkers were identified based on significant differences in the frequency of their occurrence between samples from healthy and unhealthy animals, as detected by DART MS. Overall, NMR and MS provided complementary data that showed that metabolomics is a useful approach for biomarker prospecting in poorly studied species like elasmobranchs.

Introduction

Whale sharks, Rhincodon typus Smith 1828, are circumtropical planktivorous sharks and the largest fish in the world’s oceans [1], [2], [3]. They spend their adult lives as solitary individuals migrating across the open ocean or congregating in areas of intense productivity such as coastal upwelling zones in the tropics [1], [4], where plankton densities are higher than in nutrient-limited tropical surface waters [5]. Despite their size and increasing importance as a target for ecotourism operations [2], remarkably little is known about the internal biology of this species [6].

Maintenance of a population of six whale sharks in a large public aquarium in Atlanta, USA has provided opportunities to gather new information about their biology [7]. Two animals in the collection died in 2007 after periods of 3 and 7 months of inappetence, during which they were provided with supportive nutrition and intensive veterinary care. While the onset of their illness coincided with a series of anti-parasitic treatments applied to the exhibit, none of the other 50 species in the collection died in 2007 after periods of 3 and 7 months of inappetence. The major difference between healthy and unhealthy individuals was the concentration of homarine, here reported for the first time in an elasmobranch, which was present at substantially lower concentrations in unhealthy whale sharks, suggesting that this metabolite may be a useful biomarker of health status in this species. The function(s) of homarine in sharks remain uncertain but it likely plays a significant role as an osmolyte. The presence of trimethylamine oxide (TMAO), another well-known protective osmolyte of elasmobranchs, at 0.1–0.3 mol L⁻¹ was also confirmed using both NMR and MS. Twenty-three additional potential biomarkers were identified based on significant differences in the frequency of their occurrence between samples from healthy and unhealthy animals, as detected by DART MS. Overall, NMR and MS provided complementary data that showed that metabolomics is a useful approach for biomarker prospecting in poorly studied species like elasmobranchs.
copy and direct analysis in real time (DART) mass spectrometry (MS) [13]. Metabolomics is the study of the low molecular weight (i.e. <1 kDa) molecules in a biological sample using bioanalytical and bioinformatic tools [14]. This approach has been reinvigorated recently by new technologies, allowing its application to understand metabolic perturbations such as those occurring during disease and exposure to toxicants [14,15,16,17,18]. In metabolomic studies, the progression of a disease can be observed as a trajectory deviating away from a “normal” state in principal component space [19].

Using NMR and MS metabolomic approaches, we sought to characterize variations in the metabolism of healthy and unhealthy whale sharks over a period of several months and thereby identify biomarkers of health in this elasmobranch species. We succeeded in distinguishing healthy and unhealthy animals and identified several promising biomarker compounds.

Results

1. Metabolic Profile of the Whale Shark

NMR and MS analyses of serum samples in this study represent the first examination of the physiology of the world’s largest fish. The 1H NMR spectra of serum extracts revealed the presence of a complex mixture of chemical species in 46 samples collected over a period of months from five whale sharks (Fig. 1A). Consistent with most vertebrate metabolism, the serum of whale sharks was dominated by amino acids involved in protein synthesis and hydroxy-acids involved in energy metabolism (Table 1). Yet there are some notable differences from other vertebrate groups. Trimethylamine oxide (TMAO), for example, was abundant in healthy whale shark serum samples (Fig. 1). It is a well-known osmolyte in sharks and other marine species, but is not present in appreciable quantities in mammals [20,21]. Similarly, intermediaries in the urea cycle were prominent in the metabolic profiles of whale sharks, which is perhaps not surprising given the important role of urea in the osmotic homeostasis of this and all shark species. Even more striking, homarine (N-methyl picolinic acid) is here reported for the first time from any elasmobranch species (see Appendix).

2. Metabolomic Analyses Distinguish Healthy from Unhealthy Whale Sharks

Metabolic profiles of unhealthy whale sharks were significantly different than those of healthy individuals. Pre-processing of these spectra (Fig. 1B) to remove sampling artifacts and the overwhelming influence of the most abundant metabolite, TMAO, allowed statistical evaluation of NMR spectral data by principal component analysis (PCA). Distinct separation of serum samples from the two unhealthy (Animals 1–2) versus the three healthy individuals (Animals 3–5) was evident in the first two principal components (PC1 and PC2), which together accounted for 42% of the variance in the NMR dataset (Fig. 2A). Low or negative scores on the first component (PC1) alone allowed discrimination of almost all samples originating from unhealthy individuals, except on the last day of the life of Animal 1 when veterinary intervention (intravenous dextrose) altered the metabolic profile of this individual (Fig. 3).

PCA analysis of mass spectra from 53 serum samples from all six whale sharks did not distinguish individuals based upon frequency of occurrence of individual metabolites (Fig. 2B). However, MS analyses provided tentative identification of hundreds of metabolites, of which approximately 70 were present in at least half of all samples. The lists of more commonly detected candidate compounds were then subjected to frequency analyses to extract additional patterns from the data set.

3. Small Molecules as Biomarkers

From 1H NMR spectral analysis, the heteroaromatic metabolite homarine was recognized as the component of whale shark serum contributing the greatest loading to PC1, which best separated healthy from unhealthy animals and was therefore considered a promising biomarker. The identity of this metabolite was first assigned to homarine (N-methyl picolinic acid) by liquid chromatography coupled to tandem MS and 1H NMR spectroscopy, and then confirmed by total synthesis and spectroscopic comparison of synthetic homarine and whale shark serum samples (see Appendix). Comparing peak areas of aromatic proton signals from 46 whale shark serum extracts with peak areas of an internal standard (deuterated trimethylsilylpropionate [TMSP]) of known concentration, we calculated that homarine was present in healthy whale shark serum at a concentration of approximately 1.5 mM, compared with 0.5 mM for unhealthy individuals (Fig. 4A; p<0.05 for each unhealthy animal vs. each healthy animal by ANOVA with Tukey post-hoc test; n = 2–23 samples for each individual). The concentration of homarine also declined somewhat for unhealthy Animal 1 during its time series, although this trend was not observed for the other unhealthy individual. In addition to homarine, lactate also contributed strongly to the loadings for PC1 (Fig. 5).

Unlike homarine and lactate, the variance in TMAO concentration did not distinguish all healthy from all unhealthy whale shark serum samples (Fig. 4B). Nevertheless, Animal 2 exhibited approximately 50% lower concentration of this important osmolyte relative to healthy Animals 4–5 whose serum contained 0.30–0.35 M TMAO, a difference that was found to be statistically significant by ANOVA followed by Tukey post-hoc analysis. Given that serum concentrations of TMAO were not significantly different for unhealthy Animal 1 vs. two of the healthy animals, it does not appear that TMAO is a reliable biomarker indicating whale shark health. Urea, another well-known osmolyte contributing up to 300 mOsm to shark serum, was not directly detected by 1H NMR spectroscopy due to chemical exchange of its protons with the deuterons in the solvent and so could not be considered for biomarker potential in this study.

Hundreds of metabolites were tentatively identified by their DART mass spectral data using the publically-available metabolite database METLIN (http://metlin.scripps.edu/). In contrast, matching of NMR spectra with publically-available data using an online search engine (Madison, BMRB) [22] returned few exact matches, possibly due to a combination of novel aspects of the elasmobranch metabolome and spectroscopic challenges including overlap of 1H NMR signals in whale shark serum samples. While the DART MS approach in its present implementation did not provide quantitative data related to concentration of each analyte, the analysis of presence vs. absence of compounds by MS was highly informative because of the better sensitivity and resolving power of MS compared to NMR, and because many metabolites appeared to occur at concentrations near or below the MS detection limit, such that they were apparently “absent” from some samples while detectable in others.

Overall, twenty-seven compounds including TMAO and urea were detected in at least 70% of all samples analyzed by DART MS from healthy and unhealthy individuals (Table 1). Homarine was detected by DART in 22% of the samples, but because of the potential for unwanted fragmentation during DART ionization of this type of labile N-substituted species [23], DART homarine signals were not used in our frequency analysis. Twenty-three

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additional compounds occurred in significantly different proportions of samples for healthy vs. unhealthy animals (Table 2). Decreased frequencies of ten of these biomarkers in serum samples of unhealthy individuals indicate apparent deficiencies in urea cycle, amino acid biosynthesis and catabolism, vitamin metabolism, and folate biosynthesis. Unhealthy sharks exhibited increased frequencies of 13 biomarkers that, in mammals, typically correlate with acidosis, aciduria, dysfunctional amino acid metabolism, and other indicators of abnormal metabolism (HMDB [24]) (Table 2). After Bonferroni correction for multiple comparisons, five metabolites remained significantly different between healthy and unhealthy animals; saccharopine was more frequently detected in healthy whale sharks, while 4–hydroxycyclohexylacetic acid, 2-methylglutaconic acid, 3-hexenedioic acid and 3-methylglutaconic acid were all more frequently detected in unhealthy animals.

Materials and Methods

1. General

Blood samples were collected from six whale sharks from 2006 through 2008. Animals 1 and 2 were classed as unhealthy and were sampled from October 2006 to June 2007. Animals 3, 4, 5 and 6 were classed as healthy and were sampled haphazardly from 2007 to 2008. Animals 1, 2, 5, and 6 were male. The age of the animals was unknown at the time of collection but estimated to be between 5 and 8 years for all animals in the study. Veterinary exams were conducted as described by Dove et al [6]. Briefly, individual animals were corralled by SCUBA divers into a vinyl stretcher suspended in their exhibit and sedated with hyperoxic water (120–150% saturation at 25°C) delivered towards the mouth with a flexible hose attached to a jacuzzi pump. Blood samples were then collected from the ventral caudal vein using a syringe connected to a 3.5 inch spinal needle by a 15 inch extension set and then allowed to clot in plain serum tubes (Becton Dickinson Co., Franklin Lakes NJ, USA), before being centrifuged for 10 min at 3,500 rpm (Eppendorf compact centrifuge, Hamburg, Germany) to separate the clot from the serum. Serum was drawn off in 2.0 mL aliquots, placed in CryoPro® cryovials (VWR, Westchester PA, USA) and frozen at −80°C for later analysis.

2. Sample Preparation

Whale shark serum (250 μL from each sample) was transferred to a clean 2.0 mL Eppendorf tube on ice and 500 μL of ice-cold
acetonitrile was added to precipitate proteins. The tube was immediately sealed to prevent evaporation and the sample vortexed for 15 seconds and then centrifuged for 5 min at 15,000 rpm. The supernatant was then transferred to a clean cryovial tube, and then lyophilized.

For NMR studies the samples were re-suspended in 475 µL of deuterium oxide containing 20 mM of 3-trimethylsilyl-2,2,3,3-d4-propionate (TMSP), an internal standard with respect to the resonance frequency and concentration of metabolites. For MS studies the samples were re-suspended in ultrapure water and derivatized following our previously published protocol [13].

3. NMR Metabolomics Studies

A total of 46 samples from five whale sharks (Animals 1–5) were investigated by 1H NMR spectroscopy. NMR spectra were recorded on a Bruker-Biospin AMX400 spectrometer (Bruker,
4. MS Metabolomics Studies

A total of 53 samples from all six whale sharks were investigated by mass spectrometry. MS metabolomics analysis was performed via a DART ion source (IonSense, Saugus MA, USA) coupled to an AccuTOF mass spectrometer (JEOL, Tokyo, Japan) as previously described [13]. The DART ion source was operated in positive ion mode with a helium gas flow rate of 3.0 L/min, heated to 200°C. Accurate mass spectra were acquired within the range of m/z 60–1000 with a spectral recording interval of 1.5 s. Mass drift compensation was performed after analysis of every sample using a 0.20 mM PEG 600 standard in methanol. Prior to PCA, mass spectra were normalized to the base peak intensity in Excel 2003 (Microsoft Corporation, Redmond, WA), imported as csv files, and resampled to 20,000 m/z points between 60 and 990 using the msresample function in the MATLAB Bioinformatics Toolbox.

Discussion

Metabolomic approaches revealed multivariate data patterns of serum composition that paralleled observed differences in health status of individual whale sharks, indicating that declining health in this species can be recognized by blood chemical (metabolite) profiles (Fig. 2–3). Analysis by NMR and MS led to the identification of several potential biomarkers; that is, individual compounds that vary with health status in a seemingly meaningful way. These two outcomes confirm that metabolomic methods are useful tools for studying the health of aquatic animals, consistent with previous studies [14]. Overall, metabolomics produced a tremendous data return, thus maximizing the benefit that could be extracted from samples that are so difficult to gather and which have not yet been achieved in natural environments.

After preliminary data processing, PCA of 1H NMR spectra of whale shark serum extracts showed substantial differences between healthy and unhealthy whale sharks in the overall composition and concentration of metabolites in serum (Figs. 2–3). Unhealthy animals grouped together on PC1, a clustering pattern that was largely driven by fluctuations in the aromatic region of the spectrum, which was subsequently shown to be primarily due to the influence of homarine (Fig. 5; Appendix S1). Univariate analysis of homarine measured from individual samples by NMR confirmed that differences in homarine concentration between unhealthy and healthy whale sharks were statistically significant (Fig. 4A).

Although NMR spectroscopy was useful for characterizing metabolomes in unsupervised multivariate analyses, there was little congruence between lists of candidate compounds produced by our 1H NMR experiments and major online databases of metabolites, regardless of health status of the animal from which the sample was drawn. This may have been due to the complexity of the serum mixture, the inherent insensitivity of NMR, and the exchange of protons on some metabolites by deuterons from the solvent. Given that many metabolites common to eukaryotic organisms were identified from mass spectra of these same samples...
(Tables 1–2), it seems unlikely that whale shark metabolism differs fundamentally and substantially from model organisms studied previously. The MS dataset provided more information regarding individual compounds due to greater spectral resolution, but given our frequency-based approach of analyzing MS spectral data, was less useful for distinguishing healthy and unhealthy animals by PCA (Fig. 2B). Frequency analysis of the candidate compounds, however, provided another dimension of usefulness in the MS dataset and identified a number of promising biomarker molecules (Table 2). Overall, NMR and MS approaches were complementary towards the main goal of characterizing physiological indicators of ill health in this large, metabolically complex animal species.

We expected to detect trimethylamine oxide (TMAO), based on published studies reporting that high concentrations of this protective osmolyte are apparently universal in the blood of elasmobranchs (e.g., [25]). Concentrations of TMAO varied somewhat between animals such that the animal with the longest disease progression had the most reduced serum concentration of this metabolite, but this trend was not significant enough to discriminate all healthy from both unhealthy animals (Fig. 4B), suggesting that while it may serve important functions, this compound may not be a useful biomarker of health in whale sharks. It seems likely that, due to its critical role as an osmolyte that protects against the harmful effects of urea (also retained in shark blood at high concentrations), TMAO concentrations are both high and relatively constant.

Figure 4. Differences in concentration of homarine (A) and trimethylamine-oxide (TMAO) (B) in serum samples from two unhealthy (animals 1–2) and three healthy (3–5) whale sharks. doi:10.1371/journal.pone.0049379.g004
In contrast to TMAO, homarine was determined to be a potentially useful biomarker because concentrations varied significantly between healthy and unhealthy animals (Fig. 4A). Homarine is widely distributed among marine taxa and is particularly abundant in invertebrate groups including sponges [26], gorgonians [27], corals [28], gastropods [29], bivalves [30], squids [31], holothurians [26], annelids [32], crustaceans [33,34] and ascidians [35]; it even occurs in some phytoplankton species [36]. Among these groups it has been proposed to have a similarly great diversity of functions: an osmolyte [34]; a pattern control model during development [37]; an anti fouling compound [38]; a predation deterrent [39]; an antibacterial agent [28] and an immune effector molecule [27]. Homarine has been less commonly reported from teleost fish, but does occur throughout the tissues of marine - but apparently not freshwater - fishes [40], indicating a probable osmolytic function among fishes. Since this is the first time that homarine has been reported from an elasmobranch, its specific functions in whale sharks are unclear. It may act as a protective osmolyte in a similar way as TMAO (albeit at much lower concentrations), or it may simply reflect dietary intake of the native compound or one of its precursors. Either hypothesis is supported by the lower concentrations of both homarine and urea cycle metabolites in unhealthy versus healthy sharks (Fig. 4A; Table 2). Why might homarine concentrations be lower in unhealthy whale sharks? Most likely, the anorexic nature of the illness in this case resulted in the animals not receiving necessary dietary sources of homarine or its precursors. A similar explanation may apply to the lower frequencies of precursors and products of amino acid, vitamin, and folate metabolism in unhealthy whale shark serum samples (Table 2).

In addition to uncertainty regarding the physiological significance of metabolites pinpointed due to their differential concentrations in healthy versus unhealthy whale sharks, the metabolism of whale sharks in general (e.g., involving metabolites identified in all samples, see Table 1) warrant further study. Specifically, an understanding of critical metabolites can improve conceptual models of elasmobranch metabolism, determine how it differs under conditions of chronic deep diving described by Brunnschweiler et al. [41] and Graham et al. [42]. The logical next step would be to extend these approaches to samples collected from the field, whereby biomarkers identified in the aquarium setting could be used to assess health status in wild whale shark populations (“environmental metabolomics” sensu Hines et al. [19]). This is especially relevant wherever these populations are threatened by anthropogenic factors or environmental changes. Obviously there are tremendous logistical challenges inherent in that sort of study, but the potential research

| Tentative metabolite ID | PH | PM | Class | Function (mammalian) |
|-------------------------|----|----|-------|-----------------------|
| Trimethylamine oxide (TMAO) | 1.00 | 1.00 | Aliphatic amine | Osmolyte (shown for elasmobranchs also) |
| 2-Ethoxy-2-hydroxybutyric acid | 1.00 | 0.865 | Short-chain hydroxy acid | Not a major mammalian metabolite |
| 2-Hydroxy-3-methylpentanoic acid | 1.00 | 0.919 | Short-chain hydroxy acid | Metabolite of isoleucine |
| 2-Hydroxypropanoic acid | 1.00 | 0.865 | Short-chain hydroxy acid | Endogenous but normal function uncertain |
| 5-Hydroxypentanoic acid | 1.00 | 0.865 | Short-chain hydroxy acid | Omega-oxidation product of fatty acids |
| D-Leucine acid | 1.00 | 0.865 | Short-chain hydroxy acid | Endogenous but normal function uncertain |
| Hydroxyisocaproic acid | 1.00 | 0.865 | Short-chain hydroxy acid | Metabolite of branched chain amino acids |
| Leucinic acid | 1.00 | 0.865 | Short-chain hydroxy acid | Normal function uncertain, known bacterial metabolite |
| N-Acetylglycine | 1.00 | 0.811 | Amino acid | Stable analogue of glutamine, protein synthesis |
| Urea | 1.00 | 0.892 | Amino-ketone | Osmolyte (elasmobranchs), protein catabolyte (mammals) |
| Acetic acid | 0.938 | 0.865 | Short-chain fatty acid | Metabolism of CoA, carbohydrates and fats |
| Carbon dioxide | 0.938 | 0.811 | Gas | Respiratory end-product |
| Glycolaldehyde | 0.938 | 0.865 | Alcohol/aldehyde | Precursor of Coenzyme A |
| 3,4-Dihydroxyphenylglycol | 0.875 | 0.703 | Alcohol/polyphenol | Norepinephrine metabolite |
| Cinnamaldehyde | 0.875 | 0.84 | Short-chain aldehyde | Plant metabolite (possible misidentification) |
| Dihyropterdine | 0.875 | 0.649 | Heterocyclic amine | Component of folate synthesis |
| Dimethylsulfide | 0.875 | 0.676 | Gas | Osmolyte, enzyme cofactor, signaling molecule |
| 1-deoxy-D-xylulose | 0.813 | 0.622 | Monosaccharide | Metabolite of pyridoxine, involved in vitamin B6 metabolism |
| 2,3-Dihydroxyvaleric acid | 0.813 | 0.622 | Short-chain hydroxy acid | Endogenous but normal function uncertain |
| Deoxyribose | 0.813 | 0.622 | Monosaccharide | DNA architecture, energy metabolism (via role in ATP) |
| Imidazole | 0.813 | 0.649 | Heterocyclic amine | Component of many biological molecules |
| 2-Methylacetacetic acid | 0.750 | 0.838 | Short-chain keto-acid | Intermediate in synthesis and degradation of ketones |
| 2-Oxovaleric acid | 0.750 | 0.784 | Keto/fatty acid | Valine, leucine and isoleucine metabolite |
| a-Ketosovaleric acid | 0.750 | 0.784 | Short-chain keto-acid | Precursor in levulinic acid and valine synthesis |
| Levulinic acid | 0.750 | 0.784 | Short-chain keto-acid | Component of porphyrin and chlorophyll metabolism |
| Methylacetoacetic acid | 0.750 | 0.784 | Short-chain keto-acid | Endogenous but normal function uncertain |

*PH and PM refer to the proportion of total healthy (n = 16) and unhealthy (n = 37) shark samples, respectively, from which each compound was identified. doi:10.1371/journal.pone.0049379.t001
Table 2. Candidate biomarker metabolites detected and tentatively identified by DART MS that showed a significant difference in frequency between healthy (H) and unhealthy (M) whale sharks (two-proportion z-test \( p<0.05 \)) after Bonferroni correction.

| Tentative metabolite ID | \( P_H^* \) | \( P_M^* \) | \( z \)  | \( p \)  | Corr. \( p \) | Class          | Function (mammalian)                                                                 |
|-------------------------|-------------|-------------|--------|--------|-------------|----------------|-----------------------------------------------------------------------------------|
| Saccharopine            | 0.625       | 0.189       | 3.12   | 0.001  | **0.021**   | Amino acid     | Principal normal metabolite of lysine catabolism                                   |
| L-Asparagine            | 0.688       | 0.324       | 2.46   | 0.007  | 0.164       | Amino acid     | Essential amino acid                                                                |
| Ureidopropionic acid    | 0.688       | 0.324       | 2.46   | 0.007  | 0.164       | Amino acid     | Urea cycle; CoA, pyrimidine & alanine metabolism                                    |
| D-Ornithine             | 0.688       | 0.351       | 2.26   | 0.012  | 0.275       | Amino acid     | Urea cycle; arginine & proline metabolism                                           |
| Ornithine               | 0.688       | 0.351       | 2.26   | 0.012  | 0.275       | Amino acid     | Urea cycle; component of several amino acid metabolisms                            |
| Pantetheine             | 0.688       | 0.378       | 2.07   | 0.019  | 0.440       | Tripeptide     | Intermediate in vitamin B and CoA metabolism                                        |
| N-Acetylglutamine       | 1.000       | 0.811       | 1.87   | 0.031  | 0.713       | Amino acid     | Amino acid metabolism, especially glutamine                                         |
| Dihydropteridine        | 0.875       | 0.649       | 1.68   | 0.047  | 1.076       | Heterocyclic amine | Folate biosynthesis                                                                  |
| Carbamic acid           | 0.625       | 0.378       | 1.66   | 0.048  | 1.105       | Amino acid     | Protein synthesis, amino acid biosynthesis                                         |
| Heptanoic acid          | 0.625       | 0.378       | 1.66   | 0.048  | 1.105       | Carboxylic acid |                                                                                |
| Diacetyl                | 0.313       | 0.622       | −2.07  | 0.019  | 0.446       | Ketone         | Product of malolactic fermentation                                                  |
| gamma-Butyrolactone     | 0.313       | 0.622       | −2.07  | 0.019  | 0.446       | Ketone         | \( \gamma \)-aminobutyric acid catabolite                                          |
| Oxoalan-3-one           | 0.313       | 0.622       | −2.07  | 0.019  | 0.446       | Ketone         | Urinary marker found in lactic acidosis                                             |
| 2-Ketohexanoic acid     | 0.375       | 0.703       | −2.24  | 0.013  | 0.289       | Keto acid      | Inhibits insulin homeostasis                                                       |
| 2-Methyl-3-ketovaleric acid | 0.375     | 0.703       | −2.24  | 0.013  | 0.289       | Keto/hydroxy acid | Leucine catabolite in keto-acidosis                                                 |
| 3-Methyl-2-oxovaleric acid | 0.375     | 0.703       | −2.24  | 0.013  | 0.289       | Keto acid      | Isoleucine catabolite                                                              |
| Ketoleucine             | 0.375       | 0.703       | −2.24  | 0.013  | 0.289       | Keto acid      | Neurotoxic amino acid catabolite                                                   |
| 4-Hydroxycyclohexylacetic acid | 0.188   | 0.622       | −2.09  | 0.002  | **0.043**  | Hydroxy acid   | Dysfunctional tyrosine metabolism                                                  |
| 2-Methylglutaric acid   | 0.125       | 0.568       | −2.98  | 0.001  | **0.033**  | Dicarboxylic acid | Product of metabolic acidosis found in aciduria                                    |
| 3-Hexenedioic acid      | 0.125       | 0.568       | −2.98  | 0.001  | **0.033**  | Dicarboxylic acid | FA metabolite found in aciduria                                                    |
| 3-Methylglutaric acid   | 0.125       | 0.568       | −2.98  | 0.001  | **0.033**  | Dicarboxylic acid | Catabolic leucine metabolite found in aciduria                                    |

Figure 5. Loading plot for PC1 as a means to identify NMR spectroscopic features corresponding to relevant metabolites within the serum of whale sharks. 

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returns are great and could result in some much-overdue leaps forward in our understanding of this, the world's largest fish species.

Supporting Information
Appendix S1. (DOCX)

Appendix Figure S1. Identification of homarine in whale shark serum using LC-QTOF MS. (a). Total ion chromatogram (TIC) of a partially-purified whale shark serum sample. (b). Mass spectrum of the peak at 3.76 min in (a). Product ion QTOF MS/MS spectrum of the precursor ion at m/z 138 with collision energy of 20 eV from (c); partially purified whale shark serum samples and (e); synthesized homarine. (d). Extracted ion chromatograms of ions at m/z 138 from whale shark serum samples (black curve) and synthesized homarine (blue curve). (f). Suggested fragmentation pathway for homarine. (DOCX)

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Author Contributions
Conceived and designed the experiments: AD JK JL FF MZ LG. Performed the experiments: AD HW KL JL LG JK FF. Analyzed the data: AD JK JL FF MZ MV JB. Contributed reagents/materials/analysis tools: AD FF JK LG MV. Wrote the paper: AD JK FF JL.

References
1. Colman JG (1997) A review of the biology and ecology of the whale shark. Journal of Fish Biology 51: 1219-1234.
2. Martin RA (2007) A review of behavioural ecology of whale sharks (Rhincodon typus). Fisheries Research 84: 10-16.
3. Stevens JD (2007) Whale shark (Rhincodon typus) biology and ecology: A review of the primary literature. Fisheries Research 84: 4-9.
4. de la Parra Venegas R, Hueter R, Gonzalez-Cano J, Tyminski J, Remondina J, et al. (2011) An unpreceinted aggregation of whale sharks, Rhincodon typus, in Mexican coastal waters of the Caribbean Sea. PLoS ONE Submitted.
5. Merino M (1995) Upwelling on the Yucatan Shelf: hydrographic evidence. Journal of Marine Systems 30: 101-121.
6. Dove ADM, Arnold J, Claus TMM (2010) Blood cells and serum chemistry in the world's largest fish: the whale shark Rhincodon typus. Aquatic Biology 9: 177–183.
7. Dove ADM, Coco C, Binder T, Schreiber C, Davis R, et al. (2010) Care of whale sharks in a public aquarium setting. Proceedings of the 2nd US/Russian Bilateral Conference on Aquatic Animal Health. East Lansing: Michigan State University Press.
8. Bocchetti R, Lamberci CV, Razzetti EM, Pisanielli B, Maggi C, et al. (2008) Comparison of different exposure and effect biomarkers in three elasmobranch species: Squallus acanthias, Scyliorhinus canicula and Mustelus mustelus. Marine Environmental Research 66: 99-99.
9. Karsten AH, Rice CD (2004) e-Reactive protein levels as a biomarker of inflammation and stress in the Atlantic sharpnose shark (Rhizoprionodon terraenova) from three southeastern USA estuaries. Marine Environmental Research 58: 747–751.
10. Sole M, Anto M, Baena M, Carrasnon M, Cartes JE, et al. (2010) Hispanic biomarkers of xenobiotic metabolism in eighteen marine fish from NW Mediterranean shelf and slope waters in relation to some of their biological and ecological variables. Marine Environmental Research 70: 181-188.
11. Sole M, Lobera G, Aljunevic B, Rios J, de la Parra LMG, et al. (2008) Circumneutral surfactants and lipid peroxidation levels in muscle from shelf and slope dwelling fish from the NW Mediterranean: Its potential use in pollution monitoring. Science of The Total Environment 402: 306–317.
12. Viana TF, Insacio AF, de Albuquerque C, Linde-Arias AR (2008) Biomarkers in a shark species to monitor marine pollution: Effects of biological parameters on the reliability of the assessment. Marine Environmental Research 66: 171–171.
13. Zhou M, McDonald JF, Fernandez EM (2010) Optimization of a Direct Analysis in Real Time/Time-of-Flight Mass Spectrometry Method for Rapid Serum Metabolomic Fingerprinting. Journal of the American Society for Mass Spectrometry 21: 60–75.
14. Viant MR (2007) Metabolomics of aquatic organisms: the new ‘omics’ on the block. Marine Ecology-Progress Series 332: 301–306.
15. Miller RA, Reinschuessel R, Canin MC (2007) Determination of oxytetracycline levels in rainbow trout serum on a biphenyl column using high-performance liquid chromatography. Journal of Chromatography 852: 655–636.
16. Robertson DG (2005) Metabolomics in Toxicology: A Review. Toxicological Sciences 85: 809-822.
17. Samuelsson L, Forlin L, Karlsson G, Adolfseniceri M, Larsson D (2006) Using NMR metabolomics to identify responses of an environmental estrogen in blood plasma of fish. Aquatic Toxicology 78: 341–349.
18. Viant MR (2003) Improved methods for the acquisition and interpretation of NMR metabolomic data. Biochemical and Biophysical Research Communications 310: 943–948.
19. Hines A, Oladiran GS, Bignell JP, Stentiford GD, Viant MR (2007) Direct Sampling of Organisms from the Field and Knowledge of their Phenotype: Key Recommendations for Environmental Metabolomics. Environmental Science & Technology 41: 3573–3581.
20. Treberg JR (2006) The accumulation of methylamine counteracting solutes in elasmobranchs with differing levels of urea: a comparison of marine and freshwater species. Journal of Experimental Biology 209: 860-870.
21. Zou Q, Bennison BJ, Daggett V, Murphy KP (2002) The molecular mechanism of stabilization of proteins by TMAO and its ability to counteract the effects of urea. Journal of the American Chemical Society 124: 1192-1202.
22. Utzschneider JK, Akutsu H, Doreleijers JF, Hazanu J, Ioannidou YE, et al. (2008) BioMagResBank. Nucleic Acids Research 36: D402-D408.
23. Harris GA, Hestler DM, Hampton CY, Fernandez EM (2010) Comparison of the Internal Energy Deposition of Direct Analysis in Real Time and Electrospray Ionization Time-Of-Flight Mass Spectrometry. Journal of the American Society of Mass Spectrometry 21: 855-863.
24. Wisthardt DS, Knox C, A G (2009) HMDB: a knowledgebase for the human metabolome. Nucleic Acids Research 37: D603-610.
25. Yancey PH (2005) Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. Journal of Experimental Biology 208: 2819-2830.
26. Bandaranayake WM, Des Rocher A (1999) Role of secondary metabolites and pigments in the epidermal tissues, ripe ovaries, viscera, gut contents and diet of the sea cucumber Holothuria atra. Marine Biology 133: 163-169.
27. Shapo JL, Mossler PD, Galloway SB (2007) Antimicrobial activity in the common seaweed, Leptogorgia virgulata (Cnidaria : Gorgonacea). Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 140: 65-73.
28. Slattery M, Hamann MT, McClintock JB, Perry TL, Puglisi MP, et al. (1997) Ecological roles for water-borne metabolites from Antarctic soft corals. Marine Ecology-Progress Series 161: 133-144.
29. Rosenblum ES, Tjeerdema RS, Viant MR (2006) Effects of Temperature on the accumulation of methylamine counteracting solutes in elasmobranchs with differing levels of urea. Journal of Experimental Biology 209: 860-870.
30. Carr WES, Netherton JC, Gleeson RA, Derby CD (1996) Stimulants of feeding behavior in fish: Analyses of tissues of diverse marine organisms. Biological Bulletin 190: 149–160.
31. Shirai T, Kikuchi M, Matsuo S, Inada H, Suzuki T, et al. (1997) Extractive components of the squid ink. Fisheries Science 63: 939-944.
32. Gasteiger EL, Haake PC, Gergen JA (1960) An investigation of the distribution and function of homarine (N-methyl picolinic acid). Annals of the New York Academy of Sciences 90: 622–636.
33. Dall W (1971) Role of homarine in decapod Crustacea. Comparative Biochemistry and Physiology 39: 31-41.
34. Shinagawa A, Suzuki T, Konosu S (1995) Preliminary studies on the effects of salinity on intracellular nitrogenous osmolytes in various tissues and hemolymph of the Japanese spiny lobster, Panulirus japonicus (von Schlotheim 1824). Crustaceana 68: 129–137.
35. Aicelo A, Fattorusso E, Meuna M (1996) Low molecular weight metabolites of the sea cucumber Holothuria atra. Marine Biology 133: 163–169.
36. Keller MD, Matrai PA, Kiene RP, Bellows WK (2004) Responses of coastal phytoplankton populations to nitrogen additions: dynamics of cell-associated dimethylsulfinopropionate (DMSP), glycine betaine (GBT), and homarine. Canadian Journal of Fisheries and Aquatic Sciences 61: 685-699.
39. McClintock JB, Baker BJ, Hamann MT, Yoshida W, Slattery M, et al. (1994) Homarine as a feeding deterrent in common shallow-water Antarctic lamellarian gastropod Marseniopsis mollis - a rare example of chemical defense on a marine prosobranch. Journal of Chemical Ecology 20: 2539–2549.

40. Ito Y, Suzuki T, Shirai T, Hirano T (1994) Presence of cyclic betaines in fish. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 109: 115–124.

41. Brunnschweiler JM, Baensch H, Pierce SJ, Sims DW (2009) Deep-diving behaviour of a whale shark Rhincodon typus during long-distance movement in the western Indian Ocean. Journal of Fish Biology 74: 706–714.

42. Graham RT, Roberts CM, Smart JCR (2006) Diving behaviour of whale sharks in relation to a predictable food pulse. Journal of The Royal Society Interface 3: 109–116.

43. Kind T, Fiehn O (2007) Seven Golden Rules for heuristic filtering of molecular formulas obtained by accurate mass spectrometry. BMC Bioinformatics 8: 105.

44. Polychronopoulos P, Magiatis P, Skaltsounis AL, Tilleguin F, Vardalas-Theodorou E, et al. (2001) Homarine, a common metabolite in edible Mediterranean molluscs: Occurrence, spectral data and revision of a related structure. Natural Product Letters 15: 411–418.