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Selenium protein identification and profiling by mass spectrometry: A tool to assess progression of cardiomyopathy in a whale model

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Abstract

Non-ischemic cardiomyopathy is a leading cause of congestive heart failure and sudden cardiac death in humans and in some cases the etiology of cardiomyopathy can include the downstream effects of an essential element deficiency. Of all mammal species, pygmy sperm whales (*Kogia breviceps*) present the greatest known prevalence of cardiomyopathy with more than half of examined individuals indicating the presence of cardiomyopathy from gross and histo-pathology. Several factors such as genetics, infectious agents, contaminants, biotoxins, and inappropriate dietary intake (vitamins, selenium, mercury, and pro-oxidants), may contribute to the development of idiopathic cardiomyopathy in *K. breviceps*. Due to the important role Se can play in antioxidant biochemistry and protein formation, Se protein presence and relative abundance were explored in cardiomyopathy related cases. Selenium proteins were separated and detected by multi-dimension liquid chromatography inductively coupled plasma mass spectrometry (LC-ICP-MS), Se protein

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identification was performed by liquid chromatography electrospray tandem mass spectrometry (LC-ESI-MS/MS), and Se protein profiles were examined in liver (n = 30) and heart tissue (n = 5) by SEC/UV/ICP-MS detection. Data collected on selenium proteins was evaluated in the context of individual animal trace element concentration, life history, and histological information. Selenium containing protein peak profiles varied in presence and intensity between animals with no pathological findings of cardiomyopathy and animals exhibiting evidence of cardiomyopathy. In particular, one class of proteins, metallothioneins, was found to be associated with Se and was in greater abundance in animals with cardiomyopathy than those with no pathological findings. Profiling Se species with SEC/ICP-MS proved to be a useful tool to identify Se protein pattern differences between heart disease stages in *K. breviceps* and an approach similar to this may be applied to other species to study Se protein associations with cardiomyopathy.

Keywords

Selenium; Selenoprotein; Cardiomyopathy; *Kogia breviceps*; ICP-MS; LC-ESI-MS/MS

1. Introduction

This study focuses on how selenium (Se) is associated with a primary dilated cardiomyopathy in pygmy sperm whales, *Kogia breviceps*. Studying cardiomyopathy in pygmy sperm whales is of particular interest since greater than 50 % of the whales that strand are affected by this disease. Determining the etiology may be relevant to humans since the clinically the cause(s) of non-ischemic cardiomyopathy commonly cannot be determined, even in cases that lead to end-stage heart failure or heart transplantation [1, 2]. The natural occurrence of cardiomyopathy in *K. breviceps* is greater than all other mammal species studied to date warranting further examination of factors that are associated with the onset of cardiomyopathy in this whale species. The presence of cardiomyopathy in pygmy sperm whales was first identified and described in 1985 by Bossart et al. [3]. Cardiomyopathy in *K. breviceps*, as in human, is a myocardial disease that results in deterioration of cardiomyocyte number, size and function, and diminished left and/or right ventricular function. Pygmy sperm whales present pathology of a mixed form comprising dilated and hypertrophic cardiomyopathy [1].

Selenium is an essential micronutrient for animals that is primarily acquired through diet. The major biological role of Se is in antioxidant defense, but it can be toxic at high concentrations [4]. Total Se concentration in blood reflects total Se status in an individual indicating whether Se concentrations are balanced, toxic, or deficient. However, total Se concentration provides little information about the element's bioactivity since the biological function of Se is primarily mediated by incorporation or association with proteins. The liver is the predominant site of seleno-amino acid formation, Se protein synthesis, and excretion, therefore, making liver one of the tissues of choice for which to study Se speciation [5, 6]. Additionally, the liver is affected secondarily in late stage cardiomyopathy through hepatic congestion.

Etiology of cardiomyopathy in pygmy sperm whales is currently idiopathic. Nutritional deficiencies of Se have been shown in humans, mice, dogs, and cattle to play roles in development and progression of cardiomyopathy [7–11]. Humans that suffer from Keshan disease have congestive cardiomyopathy caused by a combination of dietary deficiency of selenium and presence of Coxsackievirus or patients that are on a ketogenic diet to treat intractable epilepsy are often nutritionally deficient in Se and either experience cardiomyopathy or are at greater risk for heart disease development [9, 12, 13]. There have been relatively few studies on altered selenoprotein synthesis and research addressing whether Se roles in cardiomyopathy are correlative or causative. Many factors may contribute to development and progression of cardiomyopathy, such as genetics, infectious agents, biotoxins, and dietary intake (vitamins, Se, Hg, pro-oxidants). These factors could act singly or additively and may be interconnected to one another. Other studies have shown that multiple dietary factors can interact, such as deficiency of vitamin E and Se, to further progression of myocardium degeneration [14–16].

Many prior marine mammal studies have put forth the idea that Se status may be impacted by sequestration chemistry wherein Se binds Hg in the process of detoxifying Hg [17–19]; however the ultimate biochemical impact of this detoxification mechanism has been less studied. To properly address Se bioavailability and determine whether Se activity is truly deficient due to Hg robbing *K. breviceps* of bioactive Se leading to onset of cardiomyopathy, presence of selenoenzymes, selenoproteins, and Se-containing proteins must first be characterized in terms of abundance and form. Here, methods have been developed for the complex matrices of pygmy sperm whale liver and heart tissue to extract, purify, detect, and identify Se proteins. This study seeks to utilize mass spectrometry to identify changes in Se protein presence and abundance within animals exhibiting various states of cardiomyopathy.

2. Materials and methods

2.1. Sample collection

Since 1998, the National Institute of Standards and Technology (NIST) has cryogenically banked liver tissue from stranded individual *K. breviceps* in the National Marine Mammal Tissue Bank (NMMTB) housed at the National Institute of Standards and Technology (NIST), Charleston, SC. Liver samples obtained for the NMMTB were collected, processed, and frozen by trained field collectors during animal necropsies and stored in the specimen according to NISTIR 6279 [20]. Frozen heart samples were available from some of the same individual animals that had liver samples in the NMMTB and were donated for use in this project by the Center for Coastal Environmental Health and Biomolecular Research/National Ocean Service/National Oceanographic and Atmospheric Administration (CCEHBR/NOS/NOAA), Charleston, SC. Heart tissue samples for analytical analyses were placed in polypropylene centrifuge tubes and stored at -80°C . Fresh heart tissue that was collected at the necropsy for histology preparation and histopathological evaluation was fixed in 10 % neutral buffered formalin and tissue was sectioned at $5\text{ }\mu\text{m}$ for slide preparation. Individual animal life history and gross pathology data for this project was generated by necropsy principal investigators at collaborating institutions.

The liver for QC03LH3 pygmy sperm whale liver homogenate was collected in 1994 from a female (MMES9469SC-8) that stranded and tissues were donated to NIST, Charleston, SC by CCEHBR/NOS/NOAA for use in making an interlaboratory comparison exercise control material. QC03LH3 was used throughout method development and for Se protein identification in *K. breviceps* due to sample abundance and integrity of sample collection, homogenization, and storage.

2.2. Heart disease stage assignment

Hematoxylin and eosin (H&E) stained histology slides for each animal case were evaluated independently and blind of protein and chemical analyses data by a veterinary pathologist and assigned a heart score according to criteria put forth by Bossart et al. [1] and the Dallas cardiomyopathy criteria in humans [21]. Heart histology and gross pathology was used to categorize cardiomyopathy progression in the following three stages: no pathological findings (NPF), myocardial degeneration (MCD), and cardiomyopathy (CMP). Descriptions of each stage are provided in the Supplementary Materials along with histology images.

2.3. Sample preparation for trace element and protein analysis

Fresh frozen tissue was cryogenically homogenized to produce a uniform sample composition of fresh frozen powder for analysis. Liver samples were homogenized in class 100 clean room conditions at NIST, Charleston, SC using cryogenic procedures developed by Zeisler et al. and Pugh et al. [22, 23]. Left ventricle heart tissue was cryogenically homogenized using a bench-top 6850 Freezer/Mill (SPEX SamplePrep, Metuchen, NJ). Samples were placed in vials with a stainless steel impactor, capped, placed in the mill, submerged in LN₂, and shaken at 10Hz for 3 min. Homogenized powder was transferred into sterile polypropylene jars (Nalge Nunc International, Rochester, NY) and stored at -80 °C until analysis.

RIPA lysis buffer (Pierce, Rockford, IL) with 1X concentration Halt™ protease inhibitor cocktail and 5 mM ethylenediamine tetracetic acid (EDTA) was placed in 6 mL aliquots into polypropylene centrifuge tubes. Approximately 0.5 g homogenized sample was weighed into each centrifuge tube with buffer solution and vortexed. The centrifuge tubes were laid on ice and rocked for 15 mins. The tubes were then centrifuged for 3 min at 1500 *gn*. The supernatant was pulled off in 1.5 mL aliquots and placed into protein LoBind microcentrifuge tubes (Eppendorf, Hauppauge, NY). A Microfuge 22R (Beckman Coulter, Fullerton, CA) was used to centrifuge microcentrifuge tubes for 15 mins at 14000 *gn* and 4 °C. The supernatant from each microcentrifuge tube was filtered with a 0.2 µm PTFE membrane filter (SunSri, Rockwood, TN) into a glass auto-sampler vial. All samples were kept on ice between steps to prevent protein degradation. Protein extraction was controlled and optimized by protein concentration measurements to ensure that tissue cells were thoroughly lysed. Total protein concentration was measured at 595 nm according to the Bradford method [24] using a Coomassie Plus assay kit (Pierce) and a DU800 spectrophotometer (Beckman Coulter). Bovine serum albumin (Pierce) was used as the calibration standard.

2.4. Analytical Methods

2.4.1. Instrumentation—Total Se mass fraction measurements were made using a Thermo Electron X Series II ICP-MS (Bremen, Germany) with a standard low-volume glass impact bead spray chamber (Peltier cooled at +3 °C), concentric glass nebulizer, and operating in collision cell mode utilizing 8 % H₂ in 92 % He as the collision cell gas. Detailed total Se measurement methods and statistical analyses were described by Bryan et al. [25]. Fig. 1 outlines the multiple steps and instrumentation used for Se separation, detection, and identification. Liquid chromatography coupled to UV spectrophotometer and Thermo Elemental X7 quadrupole ICP-MS (Winsford, UK) (LC/UV/ICP-MS) were used for ⁸⁰Se separation, detection, and fraction collection. A DX 600 (Dionex, Sunnyvale, CA) ion chromatography system consisting of GP50 gradient pump, AS autosampler (cooled to 4 °C) (150 µL injection loop), and AD25 UV/Vis absorbance detector (set at 280 nm) was used for chromatographic separation. The chromatographic system was coupled to the ICP-MS by 0.010 in. internal diameter (id) PEEK tubing. Once proteins were detected by the UV absorbance detector and presence of Se was identified by ICP-MS, the chromatographic system was uncoupled from the ICP-MS and the chromatographic system was set in-line with a fraction collector (Foxy 200, ISCO, Lincoln, NE) to collect protein fractions. Liquid chromatography coupled to a Surveyor LC pump and autosampler and LTQ ESI-MS/MS (ThermoFisher, Waltham, MA) were utilized for peptide sequencing.

2.4.2. Instrument calibration methods and quality assurance—Collision cell ICP-MS working conditions were optimized with a 10 ng/g 68 element tuning solution (High Purity Standards, North Charleston, SC) and a Se calibrant prior to sample analysis and coupling to other instruments. LTQ ESI-MS/MS signal intensity was optimized using ThermoFisher Xcalibur software tune methods with angiotensin (Sigma, Sigma-Aldrich, St. Louis, MO). Commercial availability of selenoprotein standards is limited to GPx1 from bovine erythrocytes (84.5 kDa) and TrxR from rat liver (55 kDa to 67 kDa) (Sigma-Aldrich). The glutathione peroxidase standard along with procedural blanks was taken through the same analysis steps as unknown pygmy sperm whale samples. QC03LH3 and GPx standard were used throughout method development and sample analyses for method validation, reproducibility, and protein identification. The GPx standard was used to verify protein recovery along each chromatographic separation and verify that the selenoprotein could be properly identified by peptide sequence.

2.4.3. Protein separation—Protein separation steps are outlined in Fig. 1. The 1st dimension of protein separation was carried out by LC, protein presence was detected by UV/Vis, and Se containing peaks were detected by ICP-MS. Then Se containing fractions were collected from each of the 1st dimension Se containing protein peak elution times for 2nd dimension LC protein separation, ICP-MS Se detection, and fraction collection when Se species began to elute. Mobile phase compositions, chromatographic programs, and sample injection volumes are outlined in the Supplementary Materials (Table S1) for each LC separation. Liquid chromatography first dimension separation was carried out on a size exclusion column (SEC) that had an effective separation range of 1 kDa to 300 kDa since the column has notable tolerance of complex matrices. Strong anion exchange liquid chromatography (SAX/UV/ICP-MS) was used for second dimension separation additional

clean-up of Se species. Replicate injections and LC separations were performed in order to collect multiple fractions from the same sample. Strong anion exchange fractions from within each SEC Se containing protein peak were then combined and pre-concentrated using Centrifugal Filter tubes (Millipore, Billerica, MA) which have a 3 kDa molecular weight cutoff. An Avanti J-20 XPI centrifuge (Beckman Coulter) was used to spin down samples for approximately 20 min at 7,000 *gn* and 4 °C. Pre-concentrated sample that did not pass through the filter and contained proteins greater than 3 kDa was removed from the top chamber of the tube and placed in protein LoBind microcentrifuge tubes for measurement of total protein concentration or tryptic digestion.

2.4.4. Protein identification—Selenium containing protein fractions collected required protein alkylation and tryptic digestion in order to prevent formation of disulfide bridges and to obtain peptides of a suitable length for identification by LC-ESI-MS/MS. Bradford assay was used to estimate total protein concentration in each protein fraction in order to decide how much sample was required for digestion. Protein was weighed out and 0.2 % RapiGest (Waters, Milford, MA) in 50 mmol/L Tris buffer (pH 8.0) along with 200 mmol/L 1, 4-dithio-DL-threitol (DTT) (Fluka, Sigma-Aldrich) solution was added to the protein. Samples were heated at 60 °C for 30 min followed by 30 min at 37 °C. Then 200 mmol/L iodoacetamide (Sigma, Sigma-Aldrich) solution was added and samples were incubated at room temperature in the dark with vortex mixing for 1h. The alkylation reaction was stopped with the addition of 200 mmol/L DTT solution and samples were again incubated at room temperature in the dark with vortex mixing for 30 min. Trypsin (Promega, Madison, WI) was added to alkylated protein to achieve a ratio of trypsin:protein (g/g) of 1:50 to 1:100 and incubated for 20 h at 37 °C. Digestion was stopped and RapiGest was cleaved by adding 25.5 % trifluoroacetic acid (TFA) (Supelco, Bellefonte, PA) to bring sample pH < 2 prior to incubation at 37 °C for 60 min. After incubation, 0.1 % FA (aq) formic acid (Fluka, Sigma-Aldrich) was added and samples were centrifuged at 14,000 *gn* for 10 min at 4 °C to precipitate RapiGest. The supernatant was removed and transferred to protein LoBind microcentrifuge tubes which were stored frozen (−20 °C) until analysis.

Reverse Phase Liquid chromatography electrospray ionization tandem mass spectrometry (RPLC-ESI-MS/MS) was used for peptide sequencing and protein identification. Mobile phase compositions, chromatographic program, and sample injection volume are outlined in the Supplementary Materials (Table S2) for chromatographic separation of peptides. SEQUEST software (ThermoFisher, Waltham, MA) was used to compare peptide fragmentation patterns to theoretical fragmentation of peptides. The FASTA database was created by downloading all currently known protein sequences that contain the amino acid selenocysteine (U) from all species in the UniProtKB/Swiss-Prot database (downloaded from www.expasy.org on 06/10/08) since there is no protein database available for pygmy sperm whales. In addition, a custom made database was created that contained FASTA sequences from known selenoproteins and Se containing protein in humans and bovines. A list of proteins included in the custom database can be found in the Supplementary Materials (Table S3). Scoring and cutoff criteria were set in SEQUEST for database searches to decide that a MS/MS data set indicated the presence of a protein in the sample. Monoisotopic precursor and fragment mass types were allowed along with two missed cleavage sites,

cysteine alkylation, and methionine oxidation. The mass range of the peptides was 200 m/z to 6000 m/z. The XCORR versus charges state values were set at 1.50, 2.50, and 3.00; and the delta CORR cutoff value was 0.100. Maximum peptide probability was set at 0.05. This probability limit minimized the false positive rate of randomly matching a peptide in the database.

2.4.5. Selenium profiling—Liver samples from animals in the NMMTB, heart samples donated by CCEHBR/NOS/NOAA, and QC03LH3 were taken through sample preparation, separated by size exclusion chromatography, and Se species were detected by ICP-MS in a single batch to eliminate variation in ICP-MS response between batches and instrument drift was monitored over the course of the single sequence. Chromatograms were plotted for each sample to compare Se maximum peak intensities with total trace element concentrations and heart disease stage. Statistical analyses were performed using JMP 7 (SAS Institute Inc., Cary, NC) and Microsoft Excel (Redmond, Washington). Pearson's correlation analyses were carried out to determine if total trace element concentrations were linearly associated with individual Se peak maximum intensities. Analysis of variance (ANOVA) was used to analyze the relationship between individual Se peak maximum intensities and heart disease stage. Variations between heart disease stage mean maximum intensity within a peak were examined with least squares (LS) mean plots.

3. Results

3.1. Quality assurance

A single Se containing protein peak for the GPx1 standard from bovine erythrocytes had a retention time on the SEC column of approximately 9.5 min (Supplementary Materials Figure S7). The GPx1 standard from bovine erythrocytes had 97.56 % protein sequence coverage with bovine GPx1 and a list of matched peptides with their probability scores can be found in Supplementary Materials (Table S4). As a selenoprotein, glutathione peroxidase uniquely contains the selenoamino acid selenocysteine, which is coded for in a peptide sequence as a "U". The active site of GPx contains the selenocysteine residue that carries out the catalytic function of redox reactions [26]. The selenocysteine containing selenopeptide GKVLLIENVASLUGTTVR was identified in the GPx standard from bovine erythrocytes (Supplementary Materials Figure S8).

3.2. Selenium protein separation and identification

Sample separation has been identified as the limiting step in Se speciation of complex biological samples that contain a variety of Se proteins. Selenium in proteins containing selenoamino acids is covalently bound therefore the metal is less likely to separate from the protein of interest during protein separation and clean-up steps. However, Se is not covalently bound in proteins that transport Se or Se small molecules and is more easily lost during some preparations, such as SDS-PAGE, resulting in loss of detection of presence of these proteins by ICP-MS. Liquid chromatography coupled to ICP-MS was the most effective tool for separating intact Se proteins and retaining their native composition during Se separation and Se detection. Two dimensions of liquid chromatography were used for further separation and purification of Se proteins in the complex matrix of QC03LH3 pygmy

sperm whale liver homogenate to improve protein identification with tandem mass spectrometry.

Fig. 2 shows a SEC/UV/ICP-MS chromatogram of the pygmy sperm whale liver homogenate QC03LH3. Proteins and smaller molecules elute from the size exclusion column according to molecular weight resulting in larger proteins eluting first before smaller proteins. There were three prominent protein peaks containing Se from QC03LH3 that were separated by the size exclusion column. The UV peaks coinciding with Se peaks shows the absorbance for all the proteins that elute at the same time, but the selenium proteins were the only ones of interest in this study. The large Se peak with a retention time of approximately 16 minutes contained both organic and inorganic small molecule Se species and was not evaluated in this study.

Tandem mass spectrometry was used to identify proteins that were present in QC03LH3 pygmy sperm whale liver from selenium containing protein fractions separated by liquid chromatography and detected by ICP-MS. Only QC03LH3 and GPx1 standard from bovine erythrocytes were taken through the complete sequence of steps for protein identification. Table 1 provides percent sequence coverage in each protein and a list of peptides matched with the respective probability scores. Peptide matches used for identification of Se proteins in pygmy sperm whales were made assuming that a high degree of protein homology exists between species because the proteome for *K. breviceps* has not been sequenced. Protein matches between pygmy sperm whales and species for which protein databases were available indicated that peptide sequences for many proteins are likely highly conserved between species. Some Se protein sequences in *Kogia spp.* could be greatly different from those sequenced in other animals therefore preventing identification of some Se proteins in pygmy sperm whales. Additionally, the likelihood of false positive identifications is increased. Low abundance Se proteins that contribute to the ^{80}Se intensity may not have been identified since peptides for these proteins were not detectable at such low concentrations relative to greater abundance proteins. Several Se proteins were identified by a single peptide MS/MS spectrum match and should be verified by complementary means. Western blots with antibody against selenoproteins and Se-containing proteins would be the next step in protein verification.

Selenium proteins were identified in the three prominent SEC/ICP-MS protein peaks containing Se from QC03LH3 pygmy sperm whale liver homogenate (Fig. 2). Greater molecular weight selenoproteins, selenium-containing, and selenium-binding proteins were found in peaks 1 and 2; and proteins that fall into each of these Se protein classifications are identified in Table 2. Metallothioneins were identified in peak 3, which are small low molecular weight selenium-binding proteins. Select LC-ESI-MS/MS peptide spectra of Se proteins identified from SEC peaks after SAX can be found in the Supplementary Materials. Table 2 highlights the properties and functions of Se protein classes (selenoproteins, Se-containing proteins, and Se-binding proteins) identified in QC03LH3 pygmy sperm whale liver. Serum albumin was included in Table 2 since this protein can contain selenomethionine, transport small Semolecules, and was identified in several sample fractions. Serum albumin is a high-abundance protein in many tissues, including liver, making it difficult to eliminate during sample preparation.

3.3. Selenium profiling in pygmy sperm whale liver and heart tissue

Selenium species profiling was performed along with total Se and Hg concentration measurements on 30 frozen liver samples from the NMMTB and 5 frozen heart samples donated by CCEHBR/NOS/NOAA. Heart disease stages were assigned from histologic heart tissue preparations to complement 21 animals with liver samples from the NMMTB and for all 5 animal heart samples. Total Se (9.19 ± 4.00 $\mu\text{g/g}$, wet mass fraction) was positively correlated with the magnitude of certain Se protein species peaks in pygmy sperm whale livers. The maximum peak heights (cps) for peaks 2 and 3 increase significantly as total Se concentrations increase (Figs. 3B and 3C). In contrast, maximum peak heights for peak 1 do not change in relationship to increasing total Se concentrations (Fig. 3A). Fig. 4 shows a representative comparison between the SEC/ICP-MS chromatogram for liver samples from individuals at different stages of heart disease progression. Selenium protein profiles differ in liver in relationship to stage of cardiomyopathy progression. To assess the differences in Se protein peak height intensities between heart disease stages, mean individual peak intensities were calculated at each disease state as a percentage of the total sum of the three peak height intensities for that stage (Fig. 5). Animals with no pathological findings had Se protein percentages among peaks that were equivalent. Whales with cardiomyopathy had the greatest peak 3 intensities when compared to other heart disease stages. Animals with myocardial degeneration and cardiomyopathy had greater peak 3 intensities than peak 1 intensities. While peak 2 intensities increased significantly (ANOVA, $p = 0.020$) with increasing total Se, the peaks mean percentage remained constant relative to heart disease stage. Pygmy sperm whales with NPF have significantly (ANOVA, $p = 0.020$) lower total Se concentrations ((7.159 ± 1.255) $\mu\text{g/g}$, wet mass fraction) than animals with MCD ((10.502 ± 1.183) $\mu\text{g/g}$, wet mass fraction) or CMP ((11.759 ± 1.775) $\mu\text{g/g}$, wet mass fraction) [25]. Fig. 6 shows a comparison between the SEC/ICP-MS chromatograms for all heart samples at different stages of heart disease progression. Peak patterns in heart tissue were similar to liver tissue while ^{80}Se count rates were an order of magnitude lower in heart tissue than liver tissue (Figs. 2 and 6). The heart tissue chromatograms have several small Se peaks and have inferior peak resolution compared to liver tissue possibly due to poorer sample collection and storage conditions that may have allowed protein degradation, relative to specimens preserved using strict NMMTB protocols. The number and quality of heart tissue samples prevented further statistical analyses.

4. Discussion and conclusion

Cardiomyopathy in pygmy sperm whales is a chronic, progressive disease in which varying degrees of cardiac degeneration occur leading to the terminal state of advanced cardiomyopathy [1]. This appears to be the first study to assess Se protein profiles with LC/UV/ICP-MS in this mammalian species affected by a non-ischemic cardiomyopathy. For whales with MCD and CMP, Se peak patterns showed that low molecular weight proteins, such as metallothionein, were in greater abundance than animals with NPF. Once more, the relative abundance of high molecular weight Se proteins increased parallel to increasing total Se concentration. These findings may be a model for Se related non-ischemic cardiomyopathy in humans.

These Se associated proteins, glutathione peroxidase, selenium-binding protein, and metallothioneins, have critical functions in the protection from oxidative damage, metal detoxification, detecting xenobiotics, and binding xenobiotics. These protective roles are important to pygmy sperm whales because these animals are continuously exposed to oxidative stress and contaminants in the marine environment [25, 27].

4.1. Glutathione peroxidase 1

The protective effect of GPx is of particular importance when an organism is under oxidative stress [5]. Glutathione peroxidase 1 is a cellular or cytosolic enzyme that prevents lipid peroxidation of cell membranes by reducing pro-oxidants such as hydrogen peroxide and organic hydroperoxide consequently protecting cells from oxidative damage [28]. The selenoprotein glutathione peroxidase 1 protein was identified in QCO3LH3 by several separation schemes utilizing single (SEC) and two-dimensional separations (SEC and SAX). Glutathione peroxidase is the most extensively studied selenoprotein for which protein functions and structure have been widely characterized and this protein has been identified in many animal species [4]. While GPx activity has been studied in tissues from bottlenose dolphins (*Tursiops truncatus*) [29, 30] and ringed seals (*Pusa hispida*) [31], this is the first study to separate and identify GPx1 at the protein level in a marine mammal species.

4.2. Selenium binding protein 1

Selenium binding protein 1 (SBP1) was identified in QCO3LH3 pygmy sperm whale liver and selenium-binding proteins may act in sensing reactive xenobiotics in the cytoplasm [32]. Pygmy sperm whales are exposed to PCBs and high concentrations of PCBs have been measured in many other marine mammal species [33]. Rats that have been exposed to toxic coplanar polychlorinated biphenyls (PCBs) have shown up-regulation of selenium-binding protein [32]. Exposure to chemicals known to be peroxisome proliferators, such as dibutyl phthalate, Wy-14 643, and ciprofibrate, have been shown in a mouse model to decrease abundance of selenium-binding proteins [34]. Studies of the effects of different chemicals illustrate that regulation of selenium-binding protein expression may be chemical dependent. Porat et al. [35] have suggested that selenium-binding protein mediates the intracellular transport of Se. Selenium deficiency may limit SBP1 expression therefore reducing biological function of the protein.

4.3. Metallothioneins

Metallothioneins (MTs), which are Se-binding proteins, were the only Se proteins identified in peak 3 of the size exclusion chromatography separation (Fig. 2). Additional low abundance Se proteins with similar molecular weights to MTs may have been present that minimally contributed to Se intensity in peak 3, but their peptides were undetectable for identification. Metallothioneins are low in molecular weight and rich in cysteine residues. Metals bind easily to MTs due to the thiol groups (-SH) in the cysteine residues [36]. Selenium has a high binding affinity for cysteine. Metallothionein peptide fragments, identified in Se containing peak 3 and shown in Tables 1 and 2, contain several cysteine residues in each fragment. Metallothioneins are synthesized in a high capacity in tissues that uptake, store, and eliminate metals such as liver [37]. Both essential and toxic trace elements can induce MTs through chelation of cysteine residues. Metallothioneins act in maintaining

homeostasis and detoxification by restricting availability of metal cations at harmful sites [36]. Metallothioneins have been proposed as biomarkers to assess marine organisms for exposure and impact of toxic metals in the marine ecosystem [37]. Kwohn et al. [38, 39] were the first to isolate and identify MT1 and MT2 in striped dolphins (*Stenella coeruleoalba*). Metallothioneins have been detected in liver and associated with metals in several marine mammal species including sperm whales (*Physeter macrocephalus*), bottlenose dolphins, striped dolphins, pilot whales (*Globicephala melas*), narwhals (*Monodon monoceros*), Dall's porpoises (*Phocoenoides dalli*), and California sea lions (*Zalophus californianus*) [40–43].

Since the Se-binding proteins metallothionein were the only Se proteins identified in peak 3, increased relative abundance of MTs suggest their potential utility as a biomarker for onset of early stages of heart disease leading to cardiomyopathy in pygmy sperm whales. Metallothioneins have been proposed in other mammal studies as biomarkers of exposure for metal pollution [41] and findings in this study could lead to another application of MTs as biomarkers for non-ischemic cardiomyopathy. Metallothioneins have been suggested in playing a cardioprotective role by regulating metal homeostasis and anti-oxidant response [44–46]. Recently, a human study found that individuals with the MT1A genetic polymorphism are predisposed to developing cardiovascular disease when there is an imbalance between oxidant production and antioxidant defenses [47]. In our study, MT1A was specifically identified in pygmy sperm whales liver. Variations in Se protein profiles in tissues of pygmy sperm whales at different heart disease stages may lend insight into how Se protein presence and relative abundance changes throughout this type of cardiomyopathy disease progression.

4.4. Selenium concentration

Since peaks 2 and 3 increased in intensity relative to total Se concentration increase in liver, there could be similar Se incorporation mechanisms between proteins eluting in these peaks. Total Se and Hg concentrations have been shown to be closely positively correlated in liver. However, total Hg (10.452 ± 8.744 µg/g, wet mass fraction) does not have a significant correlation with the concentration of Se protein species in individual peaks in pygmy sperm whale livers (total Hg measurements were discussed in Bryan et al. [25]). This may be due to hydrogen selenide aiding in detoxifying methyl Hg and binding to inorganic Hg forming mercury selenide (HgSe) crystals, which are small inert inorganic molecules that are stored in the liver [48]. Thus, there appears to be sequestering of the bioavailable Se pool for Hg detoxification rather than for interactions involving protein formation.

4.5. Selenium species profiling in pygmy sperm whale liver and heart

Relative peak heights of Se protein species in Se profiles were related to cardiomyopathy progression in pygmy sperm whales. Differences in Se protein distribution among tissues and sub-cellular fractions have previously been identified and suggested that these proteins are involved in several metabolic pathways [49, 50]. Further biological importance of Se proteins was recognized when preferential routing of Se for formation of specific Se proteins was discovered with insufficient Se intake [49]. Selenium protein profile differences between animals with no pathological findings and animals affected by cardiac disease

indicate a potential altered metabolic pathway of protein homeostasis. Peak 2 Se protein relative intensities do not change as a function of heart disease progression suggesting that Se proteins in this peak are not related to heart disease and that peak 2 Se protein abundance increases as a consequence of total Se concentration increasing. Peak 1 intensities are not affected by total Se concentration therefore the Se proteins found in this peak could actually remain stable throughout heart disease progression even though peak 1 appears to be of greater intensity in NPF animals (Fig. 4). Given that total Se concentrations are greater in whales with CMP, accumulation of total Se in conjunction with increases in peak 3 intensities may indicate that in the presence of heart disease-correlated Se proteins found in peak 3 are up-regulated and involved in the progression of the disease state.

4.6. Selenoproteins and human heart failure

Selenoproteins are required for normal cardiac health, in particular with regards to oxidative stress, which can be associated with progressive human chronic heart failure [51]. The role of micronutrients in heart failure is also being increasingly recognized. With wider applications in higher cardiac risk populations of various forms of bariatric surgery and supplemental nutrition it becomes increasingly important to understand the pathophysiology of co-factors such as Se [52]. This is in addition to the understudied role of environmental exposures such as Se and Hg in the development or progression of heart failure. For example, a reversible dilated cardiomyopathy secondary to Se deficiency has been long-recognized (i.e., Keshan disease) [53].

Profiling Se species with SEC/ICP-MS was a useful tool in identifying differences in Se protein containing peak patterns between stages of dilated cardiomyopathy disease progression. For whales with MCD and CMP, Se peak patterns showed that low molecular weight proteins were in greater abundance than animals with NPF. Relative abundance of high molecular weight Se proteins increased parallel to total Se concentration increasing. Protein identification and profiling was the first step in gaining insight to how selenium proteins are related to cardiomyopathy in pygmy sperm whales.

Many of the factors that can contribute to onset and progression of cardiomyopathy in pygmy sperm whales may not stand alone but rather act collectively and require further investigation. Methods developed and used in this study to identify and profile Se proteins in *K. breviceps* at various stages of cardiomyopathy progression could be applied to other species that are affected by cardiomyopathy to gain further insight into disease progression and the role of Se in non-ischemic cardiomyopathy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Bossart GD, Hensley G, Goldstein JD, Kroell K, Manire CA, Defran RH, Reif JS. Cardiomyopathy and Myocardial Degeneration in Stranded Pygmy (*Kogia breviceps*) and Dwarf (*Kogia sima*) Sperm Whales. *Aquatic Mammals*. 2007; 33(2):214–222.
2. Towbin JA, Bowles NE. The failing heart. *Nature*. 2002; 415(6868):227–233. [PubMed: 11805847]
3. Bossart GD, Odell DK, Altman NH. Cardiomyopathy in Stranded Pygmy and Dwarf Sperm Whales. *Journal of the American Veterinary Medical Association*. 1985; 187(11):1137–1140. [PubMed: 4077624]
4. Arteel GE, Sies H. The biochemistry of selenium and the glutathione system. *Environmental Toxicology and Pharmacology*. 2001; 10(4):153–158. [PubMed: 21782571]
5. Behne D, Kyriakopoulos A. Mammalian selenium-containing proteins. *Annual Review of Nutrition*. 2001; 21:453–473.
6. Whanger PD. Metabolism of selenium in humans. *Journal of Trace Elements in Experimental Medicine*. 1998; 11(2–3):227–240.
7. Freeman LM, Brown DJ, Rush JE. Assessment of degree of oxidative stress and antioxidant concentrations in dogs with idiopathic dilated cardiomyopathy. *Journal of the American Veterinary Medical Association*. 1999; 215(5):644–646.
8. Bartfay WJ, Hou D, Brittenham GM, Bartfay E, Sole MJ, Lehotay D, Liu PP. The synergistic effects of vitamin E and selenium in iron-overloaded mouse hearts. *Canadian Journal of Cardiology*. 1998; 14(7):937–941. [PubMed: 9706279]
9. Bergqvist AGC, Chee CM, Lutchka L, Rychik J, Stallings VA. Selenium deficiency associated with cardiomyopathy: A complication of the ketogenic diet. *Epilepsia*. 2003; 44(4):618–620. [PubMed: 12681013]
10. Freeman LM, Brown DJ, Rush JE. Antioxidant Status in Dogs with Idiopathic Dilated Cardiomyopathy. *J Nutr*. 1998; 128(12):2768S–2770S. [PubMed: 9868262]
11. Hara S, Shoji Y, Sakurai A, Yuasa K, Himeno S, Imura N. Effects of selenium deficiency on expression of selenoproteins in bovine arterial endothelial cells. *Biol Pharmacol Bull*. 2001; 24(7):754–759.
12. Foster LH, Sumar S. Selenium in health and disease: A review. *Crit Rev Food Sci Nutr*. 1997; 37(3):211–228. [PubMed: 9143818]
13. Xia Y, Hill KE, Burk RF. Biochemical Studies of a Selenium-Deficient Population in China: Measurement of Selenium, Glutathione Peroxidase and Other Oxidant Defense Indices in Blood. *J Nutr*. 1989; 119(9):1318–1326. [PubMed: 2795246]
14. Baker MA, Cerniglia GJ, Zaman A. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. *Analytical Biochemistry*. 1990; 190(2):360–365. [PubMed: 2291479]
15. Kennedy S, Rice D. Selective morphologic alterations of the cardiac conduction system in calves deficient in vitamin E and selenium. *Am J Pathol*. 1988; 130(2):315–325. [PubMed: 3341451]
16. Kennedy S, Rice D, Davidson W. Experimental myopathy in vitamin E-and selenium-depleted calves with and without added dietary polyunsaturated fatty acids as a model for nutritional degenerative myopathy in ruminant cattle. *Research Veterinary Science*. 1987; 43:384–394.
17. Caurant F, Navarro M, Amiard J-C. Mercury in pilot whales: possible limits to the detoxification process. *Sci Total Environ*. 1996; 186(1–2):95–104. [PubMed: 8685711]
18. Koeman JH, Peeters WHM, Koudstaal Ch, Tjioe PS, Goeij J. Mercury-Selenium Correlations in Marine Mammals. *Nature*. 1973; 245(5425):385–386. [PubMed: 4593498]
19. Wang A, Barber D, Pfeiffer CJ. Protective effects of selenium against mercury toxicity in cultured Atlantic spotted dolphin (*Stenella plagiodon*) renal cells. *Archives of Environmental Contamination and Toxicology*. 2001; 41(4):403–409. [PubMed: 11598777]

20. Becker, PR., Porter, BJ., Mackey, EA., Schantz, MM., Demiralp, R., Wise, SA. National Marine Mammal Tissue Bank and Quality Assurance Program: protocols, inventory, and analytical results. NISTIR6279, USDOC, National Institute of Standards and Technology; Gaithersburg, MD: 1999.
21. Aretz HT. Myocarditis - the Dallas Criteria. Human Pathology. 1987; 18(6):619–624. [PubMed: 3297992]
22. Pugh, RS., Ellisor, MB., Moors, AJ., Porter, BJ., Becker, PR. Marine Environmental Specimen Bank: clean room and specimen bank protocols. NISTIR7389, USDOC, National Institute of Standards and Technology; Gaithersburg, MD: 2007.
23. Zeisler R, Langland JK, Harrison SH. Crygenic homogenization of biological tissues. Analytical Chemistry. 1983; 55:2431–2434. [PubMed: 6660530]
24. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein dye binding. Annals of Biochemistry. 1976; 72:248–254.
25. Bryan CE, Davis WC, McFee WE, Neumann CA, Schulte J, Bossart GD, Christopher SJ. Influence of mercury and selenium chemistries on the progression of cardiomyopathy in pygmy sperm whales, *Kogia breviceps*. Chemosphere. 2012; 89(5):556–562. [PubMed: 22704211]
26. Suzuki KT. Metabolomics of selenium: Se metabolites based on speciation studies. Journal of Health Science. 2005; 51(2):107–114.
27. Booth S, Zeller D. Mercury, Food Webs, and Marine Mammals: Implications of Diet and Climate Change for Human Health. Environmental Health Perspectives. 2005; 113(5):521–526. [PubMed: 15866757]
28. Czuczejko J, Zachara BA, Staubach-Topczewska E, Halota W, Kedziora J. Selenium, glutathione and glutathione peroxidases in blood of patients with chronic liver diseases. Acta Biochimica Polonica. 2003; 50(4):1147–1154. [PubMed: 14740001]
29. Pine MD, Greer K, Busbee D. Comparison of reactive oxygen scavenging systems between a cetacean (DKN1) and a porcine renal epithelial cell line (LLC-PK1). Comp Biochem Physiol A-Mol Integr Physiol. 2007; 147(2):550–555. [PubMed: 17389150]
30. Woshner V, Knott K, Wells R, Willetto C, Swor R, O'Hara T. Mercury and Selenium in Blood and Epidermis of Bottlenose Dolphins (*Tursiops truncatus*) from Sarasota Bay, FL: Interaction and Relevance to Life History and Hematologic Parameters. Ecohealth. 2008; 5(3):360–370. [PubMed: 19165553]
31. Vazquez-Medina JP, Zenteno-Savin T, Elsner R. Antioxidant enzymes in ringed seal tissues: Potential protection against dive-associated ischemia/reperfusion. Comp Biochem Physiol C-Toxicol Pharmacol. 2006; 142(3–4):198–204. [PubMed: 16269268]
32. Ishii Y, Hatsumura M, Ishida T, Ariyoshi N, Oguri K. Significant induction of a 54-kDa protein in rat liver with homologous alignment to mouse selenium binding protein by a coplanar polychlorinated biphenyl, 3,4,5,3',4'-pentachlorobiphenyl and 3-methylcholanthrene. Toxicol Lett. 1996; 87(1):1–9. [PubMed: 8701438]
33. Becker PR, Mackey EA, Demiralp R, Schantz MM, Koster BJ, Wise SA. Concentrations of chlorinated hydrocarbons and trace elements in marine mammal tissues archived in the U.S. national biomonitoring specimen bank. Chemosphere. 1997; 34(9–10):2067–2098. [PubMed: 9159906]
34. Giometti CS, Liang XL, Tollaksen SL, Wall DB, Lubman DM, Subbarao V, Rao MS. Mouse liver selenium-binding protein decreased in abundance by peroxisome proliferators. Electrophoresis. 2000; 21(11):2162–2169. [PubMed: 10892727]
35. Porat A, Sagiv Y, Elazar Z. A 56-kDa Selenium-binding Protein Participates in Intra-Golgi Protein Transport. Journal of Biological Chemistry. 2000; 275(19):14457–14465. [PubMed: 10799528]
36. Kang YJ. Metallothionein redox cycle and function. Experimental Biology and Medicine. 2006; 231(9):1459–1467. [PubMed: 17018867]
37. Sarkar A, Ray D, Shrivastava AN, Sarker S. Molecular biomarkers: Their significance and application in marine pollution monitoring. Ecotoxicology. 2006; 15(4):333–340. [PubMed: 16676218]
38. Kwohn YT, Okubo A, Hirano H, Kagawa H, Yamazaki S, Toda S. Primary structure of striped dolphin renal metallothionein-II. Agricultural and Biological Chemistry. 1988; 52(3):837–841.

39. Kwohn YT, Yamazaki S, Okubo A, Yoshimura E, Tatsukawa R, Toda S. Isolation and characterization of metallothionein from kidney of striped dolphin, *Stenella coeruleoalba*. *Agricultural and Biological Chemistry*. 1986; 50(11):2881–2885.
40. Decataldo A, Di Leo A, Giandomenico S, Cardellicchio N. Association of metals (mercury, cadmium and zinc) with metallothionein-like proteins in storage organs of stranded dolphins from the Mediterranean sea (Southern Italy). *Journal of Environmental Monitoring*. 2004; 6(4):361–367. [PubMed: 15054547]
41. Das K, Debacker V, Bouqueneau JM. Metallothioneins in marine mammals. *Cellular and Molecular Biology*. 2000; 46(2):283–294. [PubMed: 10774920]
42. Wagemann R, Hunt R, Klaverkamp JF. Subcellular distribution of heavy metals in liver and kidney of a narwhal whale (*Monodon monoceros*): An evaluation for the presence of metallothionein. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology*. 1984; 78(2):301–307.
43. Ikemoto T, Kunito T, Anan Y, Tanaka H, Baba N, Miyazaki N, Tanabe S. Association of heavy metals with metallothionein and other proteins in hepatic cytosol of marine mammals and seabirds. *Environmental Toxicology and Chemistry*. 2004; 23(8):2008–2016. [PubMed: 15352491]
44. Ceylan-Isik AF, Zhao P, Zhang BF, Mao XY, Su GH, Ren J. Cardiac overexpression of metallothionein rescues cardiac contractile dysfunction and endoplasmic reticulum stress but not autophagy in sepsis. *Journal of Molecular and Cellular Cardiology*. 48(2):367–378.
45. Liu QJ, Wang GJ, Zhou GH, Tan Y, Wang XL, Wei W, Liu LC, Xue WL, Feng WK, Cai L. Angiotensin II-induced p53-dependent cardiac apoptotic cell death: Its prevention by metallothionein. *Toxicol Lett*. 2009; 191(2–3):314–320. [PubMed: 19808082]
46. Ye G, Metreveli NS, Ren J, Epstein PN. Metallothionein prevents diabetes-induced deficits in cardiomyocytes by inhibiting reactive oxygen species production. *Diabetes*. 2003; 52(3):777–783. [PubMed: 12606520]
47. Giacconi R, Kanoni S, Mecocci P, Malavolta M, Richter D, Pierpaoli S, Costarelli L, Cipriano C, Muti E, Mangialasche F, Piacenza F, Tesei S, Galeazzi R, Theodoraki EV, Lattanzio F, Dedoussis G, Mocchegiani E. Association of MT1A haplotype with cardiovascular disease and antioxidant enzyme defense in elderly Greek population: comparison with an Italian cohort. *The Journal of Nutritional Biochemistry*. In Press, Corrected Proof.
48. Falnoga I, Tusek-Znidaric M, Stegnar P. The influence of long-term mercury exposure on selenium availability in tissues: An evaluation of data. *Biometals*. 2006; 19(3):283–294. [PubMed: 16799866]
49. Behne D, Hilmert H, Scheid S, Gessner H, Elger W. Evidence for specific selenium target tissues and new biologically important selenoproteins. *Biochimica Et Biophysica Acta*. 1988; 966(1):12–21. [PubMed: 3390461]
50. Behne D, Scheid S, Kyriakopoulos A, Hilmert H. Subcellular-distribution of selenoproteins in the liver of the rat. *Biochimica Et Biophysica Acta*. 1990; 1033(3):219–225. [PubMed: 2317500]
51. de Lorgeril M, Salen P. Selenium and antioxidant defenses as major mediators in the development of chronic heart failure. *Heart Fail Rev*. 2006; 11(1):13–17. [PubMed: 16819573]
52. McKeag NA, McKinley MC, Woodside JV, Harbinson MT, McKeown PP. The Role of Micronutrients in Heart Failure. *J Acad Nutr Diet*. 2012; 112(6):870–886. [PubMed: 22709814]
53. Ge KY, Yang GQ. The epidemiology of selenium deficiency in the etiologic study of endemic diseases in China. *American Journal of Clinical Nutrition*. 1993; 57(2):259–263.

Fig. 1.

Flow chart of selenium species separation, detection, and identification methods. 1st dimension LC – size exclusion chromatography (SEC); 2nd dimension LC – strong anion exchange (SAX).

Fig. 2. SEC/UV/ICP-MS chromatogram (^{80}Se intensity) of QC03LH3 pygmy sperm whale liver homogenate overlaid with UV absorption profile. Numbers indicate Se containing protein peaks.

Fig. 3.

Effect of the total selenium concentration on the intensity (cps) of selenium species in individual peaks in pygmy sperm whale livers ($n = 30$). Relationship between maximum peak intensity and total Se concentration for peaks 1 (A), 2 (B), and 3 (C). Data points are fitted with linear trend lines and data was collected in a single day.

Fig. 4. Pygmy sperm whale liver SEC/ICP-MS ^{80}Se profiles for are presentative individual at each heart disease stage; no pathological findings (NPF), myocardial degeneration (MCD), cardiomyopathy (CMP). Inset shows protein peak region of the SEC/ICP-MS chromatogram.

Fig. 5.

Pygmy sperm whale liver Se protein distribution in each protein peak as a percentage (mean $\pm$ SE) of the sum of the three Se containing protein peak heights relative to heart disease stage (*n* = number of whales at disease stage); no pathological findings (NPF), myocardial degeneration (MCD), cardiomyopathy (CMP).

Fig. 6. SEC/ICP-MS ^{80}Se profiles for 5 individual pygmy sperm whale hearts with different heart disease stages; no pathological findings (NPF), myocardial degeneration (MCD), cardiomyopathy (CMP).

Table 1

QC03LH3 Se protein peptide matches, species, % protein sequence coverage, and peptide probability (P) value.

Protein	Species	% protein sequence coverage	Peptides	P value
Glutathione peroxidase 1	Bovine	54.63%	CEVNGEK	8.32E-05 *
			SAAAAAAPP	1.38E-04
			NEELNCLK	5.70E-05 *
			FITWSPVCR	3.56E-04 *
			FLVGPDPVPVR	1.22E-05
			FLVGPDPVPVRR	5.88E-05 *
			GLVVLGPPCNQFGHQENAKNEELNCLK	1.06E-11
			YVRPGGGFEPNFMFLFEK	5.13E-07
			FLTIDIEPDITLLSQGASA	8.23E-04
			AHPLFAFLR	3.24E-06
Glutathione S-transferase A1	Human	30.35%	GLVVLGPPCNQFGHQENAK	5.56E-11 *
			VLLIENVASLUGTTVR	2.93E-09 *
			YVRPGGGFEPNFMFLFEK	2.62E-09 *
			NDVAWNFEK	3.79E-07 *
			NDVAWNFEK	9.04E-06
			DYTEMNDLQKR	5.34E-07
			EALPTPSDDATALMTDPK	1.09E-10
			YVRPGGGFEPNFMFLFQK	1.44E-05
			AILNYIATKYNLYGKDMK	4.83E-09
			FQDGLTLTYSNAILR	4.85E-09
Glutathione S-transferase P	Long-tailed hamster	7.62%	FEDGDLTLTYSNAILR	5.63E-07
			PPYTITYFPVVR	1.35E-04

Protein	Species	% protein sequence coverage	Peptides	P value
Glyceraldehyde-3-phosphate dehydrogenase	Bovine	37.24%	AENGKLVINGK	2.47E-03 *
			LEKPAKYDEIKK	3.50E-05 *
			GAAQNIIPASTGAAG	1.09E-07 *
			VVDLMVHMASKE	7.73E-05 *
			IVSNASCTTNCLAPLAK	1.11E-09 *
Glyceraldehyde-3-phosphate dehydrogenase	Human	20.00%	AITIFQERDPANIK	3.51E-04
			VPTPNVSVVDLTCR	4.77E-04 *
			LTCMAFRVPTPNVSVVDLTCR	3.09E-05 *
			RVIISAPSAADAPMFVMGVNHEK	8.69E-05
			WGDAGAEYVVESTGVFTTMEK	5.00E-13 *
Glyceraldehyde-3-phosphate dehydrogenase	Human	20.00%	VKVGNGFGR	4.73E-05 *
			LISWYDNEFGYSNR	2.93E-06 *
			RVIISAPSAADAPMFVMGVNHEK	2.36E-05 *
			WGDAGAEYVVESTGVFTTMEK	9.63E-10
			GASDKCSCCA	2.19E-06
Metallothionein-1A	Bovine	29.51%	CAQGCVCCK	9.66E-04
			CAQGCVCCKGASDK	1.85E-06
			CAQGCVCCKGASDKCSCCA	2.65E-13
			GASDKCSCCA	3.89E-07 *
			SCCSCCPAECEK	1.24E-05 *
Metallothionein-2	Human	16.39%	SCCSCCPAECEK	4.34E-06
			CAQGCICKGSDKCSCCA	2.87E-08
			YGPMEELVIEK	5.14E-06 *
			GGPVQVLEQDELK	1.26E-06
			GGPVQVLEQDELK	1.26E-06

Protein	Species	% protein sequence coverage	Peptides	P value
			TKLLLLPSLISSR	8.77E-07 *
	Human	7.84%	GGPVQVLEDELK	6.94E-06 *
			LTGQLFLGGSIVK	3.66E-07
			EEIVYLPICYR	4.62E-06 *
Thioredoxin reductase 1	Human	1.54%	FLIATGERPR	2.79E-04 *
3-mercaptopyruvate sulfurtransferase	Human	9.76%	AGQPLQLLDASWYLPK	2.81E-08
			ALVSAQWVAEALR	7.53E-05 *

* P value was calculated from the custom made human and bovine protein database since peptides associated with these values were not identified from the SwissProt database.

Table 2

Properties and functions of Se proteins identified in QC03LH3 pygmy sperm whale liver.

Protein	Protein Class	Species	Sequence Length	MW (kDa)	Known function
Glutathione peroxidase 1	Selenoprotein	Bovine	205 AA	22.7	protects hemoglobin in red blood cells from oxidative breakdown
		Human	201 AA	21.9	
		Common marmoset	201 AA	21.8	
		Mouse	201 AA	22.3	
		Pig	206 AA	22.6	
		Rabbit	200 AA	21.9	
Glutathione S-transferase A1	Se-binding	Bovine	222 AA	25.5	conjugation of reduced glutathione to hydrophobic electrophiles
Glutathione S-transferase P	Se-binding	Bovine	210 AA	23.6	conjugation of reduced glutathione to hydrophobic electrophiles; aids in detoxification with xenobiotic metabolism
		Long-tailed hamster	210 AA	23.6	
		Pig	207 AA	23.5	
Glyceraldehyde-3-phosphate dehydrogenase	Se-binding	Bovine	333 AA	35.9	involved in metabolic switch under oxidative stress allowing cells to produce more NADPH
		Human	335 AA	36.1	
		Greater Egyptian jerboa	363 AA	39.4	
Metallothionein-1A	Se-binding	Bovine	61 AA	6.0	high cysteine residue content binds heavy metals
Metallothionein-2	Se-binding	Human	61 AA	6.0	high cysteine residue content binds heavy metals
Metallothionein-3	Se-binding	Human	68 AA	6.9	binds heavy metals
		Wild yak	68 AA	6.9	
Metallothionein-4	Se-binding	Dog	62 AA	6.2	binds heavy metals
Phospholipid hydroperoxide glutathione peroxidase	Selenoprotein	Human	197 AA	22.2	protects cells against membrane lipid peroxidation and oxidative damage
Selenium-binding protein 1	Se-binding	Bovine	472 AA	52.6	selenium-binding protein; involved in sensing reactive xenobiotics in the cytoplasm
		Human	472 AA	52.4	
Serum albumin	Se-containing	Bovine	607 AA	69.3	most abundant protein in plasma, regulates colloidal osmotic pressure of blood, acts as a plasma carrier by non-specific binding
		Human	609 AA	69.4	

Protein	Protein Class	Species	Sequence Length	MW (kDa)	Known function
Thioredoxin reductase 1	Selenoprotein	Human	649 AA	70.9	reduces thioredoxin using NADPH
3-mercaptopyruvate sulfurtransferase	Se-binding	Human	297 AA	33.2	transfers sulfur-containing groups (Se can substitute) to thiol compounds; participates in cysteine metabolism