Evidence of a lipid signaling molecule involved in the stress response in the Pacific oyster, *Crassostrea gigas*

Lipid signaling during oyster stress

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ABSTRACT

The lipid signaling molecule, ceramide, is a key component of the vertebrate stress response, however, there is limited information concerning its role in invertebrate species. In order to identify genes involved in ceramide metabolism in bivalve molluscs, Pacific oyster genomic resources were examined for genes associated with ceramide metabolism and signaling. Several genes were identified including full-length sequences characterized for *serine palmitoyltransferase-1, 3-ketodihydrosphingosine reductase, acid ceramidase,* and *ceramide glucosyltransferase*. Genes involved in ceramide synthesis and metabolism are conserved across taxa in both form and function. Expression analysis as assessed by quantitative PCR indicated all genes were expressed at high levels in gill tissue. The role of the ceramide pathway genes in the invertebrate stress response was also explored by measuring expression levels in juvenile oysters exposed to *Vibrio vulnificus*. A gene involved in hydrolytic breakdown of ceramide, *acid ceramidase*, was upregulated in a bacterial challenge, suggesting a possible role of ceramide in the invertebrate stress and immune responses. *In silico* and laboratory results support that Pacific oysters have the basic components of the ceramide metabolism pathway. These results also indicate that ceramide may have analogous functions in vertebrates and invertebrates. The gene expression pattern of *acid ceramidase* in response to bacterial exposure especially supports that ceramide and sphingolipid metabolism may be involved in the oyster's stress and/or immune responses.

KEYWORDS: oyster; *Crassostrea gigas*; stress; lipid; bacteria

INTRODUCTION

Ceramide is a sphingolipid that serves as an important signaling molecule for a variety of cellular processes including differentiation, proliferation, inflammation, and apoptosis [reviewed in 1 and 2]. Different stimuli promote either *de novo* synthesis of ceramide or its catabolic generation from sphingolipids [1, 2]. The diversity of processes in which ceramide plays a role as a signaling molecule indicates its importance across a variety of life stages and environmental conditions. For example, the accumulation of ceramide can halt embryonic development [3], inhibit insulin signaling [4], and promote apoptosis during cellular stress [5]. The production of ceramide can be triggered by multiple pathways and is sensitive to exogenous stressors [6, 5]. In sea bass (*Diecentrarchus labrax*), changes in intracellular ceramide levels in gill tissue are associated with abrupt shifts in environmental salinity [7]. Leukemia cells exposed to various exogenous stressors (ionizing radiation, hydrogen peroxide, UV radiation, and heat shock) showed elevated levels of ceramide and increased apoptosis [8].

Ceramide metabolism has also been associated with immune-related processes. Cytokines can trigger sphingomyelin hydrolysis, leading to increased production of ceramide, suggesting that ceramide could propagate cytokine signaling [2]. Ceramide also plays a key role in the inflammatory response in *Homo sapiens* dermal fibroblasts by stimulating interleukin-1 mediated prostaglandin E2 production [9].

While the role of ceramide as a signaling molecule in response to stress has been well studied in mammalian systems, there is little information regarding the function and metabolism of ceramide in invertebrates. Given the range of environmental conditions experienced by intertidal species, ceramide signaling could be a key component in the cellular response to these environmental changes. The primary goal of this study was to

characterize genes associated with ceramide metabolism in the intertidal mollusc, the Pacific oyster (*Crassotrea gigas*). Using an *in silico* approach, numerous genes associated with ceramide metabolism were identified and complete coding sequences were isolated for select genes. To provide insight into the functional role of ceramide metabolism in the invertebrate stress response, juvenile *C. gigas* were exposed to the marine bacterium *Vibrio vulnificus* and the expression levels of four genes involved in the ceramide pathway were assessed.

METHODS

Gene discovery

Genes involved in *Crassostrea gigas* ceramide metabolism were identified using publicly available sequence data. Specifically, short read sequences from *C. gigas* larvae complementary DNA (cDNA) libraries (GenBank Accession Number SRX032364) as well as all expressed sequence tags (ESTs) were downloaded from NCBI (www.ncbi.nlm.nih.gov). All sequences were quality trimmed and *de novo* assembled using CLC Genomics Workbench v3.7 (CLC bio, Katrinebjerg, Denmark). Consensus sequences from short read and EST assemblies were compared to the UniProtKB/Swiss-Prot database (http://www.uniprot.org) using NCBI's BLASTx algorithm [10]. Sequences having a top blast hit with an e-value less that 1E-30 were inspected for genes associated with ceramide metabolism. Individual sequence alignments were performed to determine percent coverage and sequence similarity (Geneious Pro v. 4.8.5 [11]).

Gene sequencing

93 Juvenile *Crassostrea gigas* (mean length = 11.4 cm) were obtained from Taylor
94 Shellfish Farms, Inc. (Quilcene, WA). Tissues (gill, mantle, adductor muscle, and digestive
95 gland) were dissected from *C. gigas* using sterile techniques and stored in RNAlater
96 (Ambion, Carlsbad, CA). RNA isolation was carried out using Tri-Reagent (Molecular
97 Research Center, Cincinnati, OH) per the manufacturer's protocol. Following RNA isolation,
98 samples were treated with the Turbo DNA-free Kit, rigorous protocol (Ambion) to remove
99 potential genomic DNA carry-over. All samples were evaluated to insure genomic DNA was
100 absent by performing quantitative polymerase chain reaction (qPCR) on DNAsed RNA
101 samples. RNA samples were reverse transcribed using M-MLV reverse transcriptase
102 according to manufacturer's protocol (Promega, Madison, WI).
103 For genes where the putative open reading frame could be determined based on
104 sequence alignments, PCR primers were designed to amplify entire coding regions (Primer
105 3 in Geneious Pro v. 4.8.5; [12, 11]) (Table 1). PCR reactions (25µl) using cDNA from gill
106 tissue were carried out with 12.5 µL 2x Apex RED Taq Master Mix (Genesee Scientific, San
107 Diego, CA), 8.5 µL nuclease-free water, 0.5 µL of 10 µM forward and reverse primers
108 (Integrated DNA Technologies, Coralville, IA), and 3 µL cDNA template. Thermal cycling
109 parameters were as follows: 95°C for 10 minutes; 40 cycles of: 95°C for 30 seconds, 55°C
110 for 30 seconds, and 72°C for 30 seconds; 72°C for 10 minutes. PCR products were
111 separated on agarose gels, checked for expected amplicon size, excised, cloned in pCR 2.1-
112 TOPO Vector, and transformed in to One Shot Top10 chemically competent cells using the
113 TOPO TA Cloning Kit (Invitrogen, San Diego, CA). Plasmid DNA was isolated from bacterial
114 cultures using the Qiagen MiniPrep Kit, following the manufacturer's protocol (Qiagen,
115 Valencia, CA) and sequenced at the High Throughput Genomics Unit (University of

Washington) using vector-specific primers (Invitrogen).

Sequences were trimmed to their open reading frame and translated to their amino acid sequences (Geneious Pro v. 4.8.5 [11]). Sequence alignments were performed using ClustalX v. 2.1 [13].

Protein phylogeny

Using NCBI's HomoloGene database, sequences for corresponding proteins in *Homo sapiens*, *Mus musculus*, *Danio rerio*, *Xenopus tropicalis* and *Caenorhabditis elegans* were downloaded where available. Using the PhyML plugin in Geneious [14, 11], maximum likelihood phylogenetic trees of the protein sequences were constructed based on the James-Taylor-Thornton (JTT) model and bootstrapped 100 times [15, 14].

Quantitative PCR

DNA-free RNA was reverse transcribed to cDNA as described above. qPCR was performed using 1µL of cDNA in a 25µL reaction containing 12.5 µL of 2x Immomix Master Mix (Bioline, London, UK), 0.5 µL of 10 µM forward and reverse primers, 1.0 µL 50µM SYTO13 (Invitrogen), and 9.5 µL nuclease-free water. Primers used for qPCR are listed in Table 1. Thermal cycling and fluorescence detection was performed using a CFX96 Real-Time Detection System (Bio-Rad, Hercules, CA). Cycling parameters were as follows: 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds, 55°C for 15 seconds, 72°C for 30 seconds. Immediately after cycling, a melting curve protocol was run to verify that a single product was generated in each reaction.

Average Ct (fluorescence-based cycle threshold) values across replicates and

average gene efficiencies were calculated with PCR Miner (http://www.miner.ewindup.info/version2 [16]). Gene expression (R_0) was calculated based on the equation $R_0 = 1/(1+E)^{Ct}$, where E is the average gene efficiency and Ct is the cycle threshold for fluorescence. All expression values were normalized to expression of *elongation factor 1α (*GenBank Accession Number AB122066*)*. All qPCRs were run in duplicate and significant differences in expression were determined via pairwise t-tests in R [17] with α=0.05.

Bacterial Challenges

For bacterial challenges, *Vibrio vulnificus* was grown in 400 mL culture medium (1x standard Luria-Bertani broth with an additional 1% NaCl) at 37°C for 18 hours at 150 rpm. The culture was then centrifuged for 10 minutes at 4300 rpm (25°C), the supernatant was removed and the pelleted bacteria were resuspended in 50 mL non-sterile of seawater. Eight oysters held in 8 L of seawater were inoculated with *V. vulnificus* at an initial concentration of $4.56x10^{18}$ CFU/L via a 3 hour immersion bath. Control oysters (n=8) were likewise placed in 8L of seawater. Following exposure, oysters were harvested aseptically and gill tissue was dissected and immediately frozen at -80°C. RNA isolation, reverse transcription and quantitative PCR analysis was carried out as described.

RESULTS

A total of 23 sequences associated with ceramide metabolism were identified by analyzing publicly available *Crassostrea gigas* sequences (Table 2). A majority of the

genes are either involved in *de novo* synthesis, catabolic generation, or enzymatic breakdown of ceramide (Figure 1). Most sequences were derived from contigs generated by assembling short read sequences (see supplemental data S1). Of the 23 sequences, 4 were selected for further characterization based on the percent of putative open reading frame identified. These four genes include *serine palmitoyltransferase-1* (Cg-*sptlc1)*, *3-ketodihydrosphingosine reductase* (Cg-*3KDSR)*, *acid ceramidase* (Cg-*AC),* and *ceramide glucosyltransferase* (Cg-*GlcCer).* Based on amino acid alignments, complete nucleotide open reading frames were obtained for Cg-*sptlc1* (GenBank Accession Number JN315146), Cg-*3KDSR* (GenBank Accession Number JN315143), and Cg-*AC* (GenBank Accession Number JN315144). Cg-*GlcCer* (GenBank Accession Number JN315145) is missing a portion of the 3' end of the nucleotide sequence as determined from alignments with full-length sequences in other species.

Serine palmitoyltransferase-1

The open reading frame of Cg-*sptlc1* is 1404 bp and is most similar to *sptlc-1* in *Xenopus tropicalis* (GenBank Accession Number NM_001079574) with 71% nucleotide sequence similarity. The next most similar sequence is from the hemichordate, *Saccoglossus kowalevskii* (GenBank Accession Number XM_002730516, with 70% identity). At the amino acid level Cg-*sptlc1* is most similar to serine palmitoyltransferase 1 in the Sumatran orangutan, *Pongo abelii* (GenBank Accession Number Q5R9T5). Based on alignments at the deduced amino acid level, Cg-*sptlc1* shares 59.8% pairwise identity with the *H. sapiens* homolog and 51.0% pairwise identity over 475 amino acids with *Caenorhabditis elegans* Sptlc1 (Figure 2).

185 The highest level of Cg-*sptlc1* gene expression was detected in gill tissue, followed
186 by digestive gland, mantle, and then adductor muscle (data not shown). Expression levels
187 in gill tissue were 40 times higher the levels in adductor muscle tissue. Cg-*sptlc1*
188 expression was not significantly altered in gill tissue from oysters exposed to *Vibrio*,
189 compared to controls. (p=0.068; Figure 6).
190
191 *3-ketodihydrosphingosine reductase*
192 The Cg-*3KDSR* open reading frame is 1129 bp and is most similar to the *Rattus*
193 *norvegicus 3KDSR* sequence (GenBank Accession Number NM_001108342) with a sequence
194 identity of 68%. The second most similar sequence is *3KDSR* from *Saccoglossus kowalevskii*
195 (GenBank Accession Number SM_002740331, 76%). The amino acid translation of Cg-
196 *3KDSR* is most similar to *Mus musculus* 3KDSR (GenBank Accession Number Q6GV12). The
197 *Crassostrea gigas* amino acid sequence shares 50.8% identity to the corresponding
198 homolog in *Homo sapiens* (Figure 3). Based on the derived amino acid sequence of Cg-
199 *3KDSR,* the catalytic site and NADH/NADPH binding site [18] are conserved in oysters
200 (Figure 3). Gene expression of Cg-*3KDSR* was highest in gill tissue with expression levels
201 over 1000 times higher compared to other tissues (data not shown). Cg-*3KDSR* gene
202 expression in *Vibrio*-exposed oysters was not different from controls (p=0.079; Figure 6).
203
204 *Ceramide glucosyltransferase*
205 Cg-GlcCer (1124bp) is most similar to ceramide glucosyltransferase from the human
206 body louse, *Pediculus humanus corporis* (GenBank Accession Number SM_002431306,
207 sequence similarity of 66%), followed by *Xenopus laevis* UDP-glucose ceramide

glucosyltransferase (GenBank Accession Number NM_001090475, sequence similarity of 66%). The translated amino acid sequence is most similar to *Xenopus tropicalis* ceramide glucosyltransferase (GenBank Accession Number Q5BL38). *Crassostrea gigas* and *Homo sapiens* share a 45.9% pairwise amino acid identity over 396 residues in the alignment, while *Caenorhabditis elegans* and *C. gigas* share 40.9% pairwise identity over 468 residues (Figure 4).

Cg-*GlcCer* had a similar expression profile across tissues to Cg-*sptlc1*, the highest expression being in the gill, followed by digestive gland, mantle and adductor (data not shown). The Cg-*GlcCer* gene was not expressed differently in *Vibrio*-exposed oysters compared to control oysters (p=0.47; Figure 6).

Acid ceramidase

The open reading frame for Cg-*AC* is 1170 bp in length and was most similar to the gene BRF 7-G7 in *Sebastes schlegelii* (Schlegel's black rockfish, GenBank Accession Number AB491143), with a sequence similarity of 67%. The translated amino acid sequence for *Crassostrea gigas* is the most similar to *Rattus norvegicus* acid ceramidase (GenBank Accession Number Q6P71). The Cg-*AC* amino acid sequence shares 46.6% pairwise identity over 402 residues in the alignment with *Caenorhabditis elegans* and 49.4% identity with the *Homo sapiens* sequence over 398 residues (Figure 5).

Cg-*AC* was expressed the most in the gill tissue followed by digestive gland, mantle, and adductor (data not shown). The expression of Cg-*AC* was significantly higher in *Vibrio*-exposed oysters compared to controls (p=0.045; Figure 6).

231 All four genes showed similar phylogenetic topologies (Figure 7), with the amino
232 acid sequences clustering into distinct invertebrate and vertebrate lineages. When the
233 *Caenorhabditis elegans* sequence was available and included in the phylogeny, it clustered
234 with the *Crassostrea gigas* sequence with a bootstrap value of 100%. *Homo sapiens* and
235 *Mus musculus* sequences always clustered together with a bootstrap of 100%.
236
237 DISCUSSION
238 This study identified a suite of genes associated with ceramide metabolism in the
239 Pacific oyster, including the direct sequencing and characterization of *serine*
240 *palmitoyltransferase-1* (Cg-*sptlc1*), *acid ceramidase* (Cg-*AC*), *3-ketodihydrosphingosine*
241 *reductase* (Cg-*3KDSR*), and *ceramide glucosyltransferase* (Cg-*GlcCer*). These data provide an
242 important resource for further studies that focus on the role of ceramide in the
243 environmental response in invertebrates. While well studied in vertebrate systems, there
244 have been only a few recent studies that focus on ceramide metabolism and signaling in
245 molluscs [see 19-21].
246 Numerous genes associated with ceramide metabolism are conserved across distant
247 taxonomic lineages. In vertebrates, the genes described here are directly responsible for
248 synthesis of ceramide (*sptlc1, 3KDSR*; Figure 1) and generation of sphingolipids from
249 ceramide (*AC* and *GlcCer*). *In silico* analysis of the *Crassostrea gigas* transcriptome shows
250 that there are a number of other genes in these ceramide metabolism pathways (Table 2).
251 In fact, almost all the genes coding for enzymes necessary for *de novo* ceramide synthesis
252 were identified, suggesting a conservation of this metabolic pathway in *C. gigas*.
253 Additionally, a number of enzymes responsible for transformation of ceramide into other

lipid products were identified including ceramide kinase, ceramide synthase , and sphingomyelin synthase . A variety of caspases, TNF superfamily receptors, RIP (receptor-interacting serine/threonine-protein) and FADD (Fas-associated protein with death domain) subunits of the TNFα (tumor necrosis factor) receptor, which are key components of the cellular stress and apoptotic responses, were also identified in public databases . Several genes known to be involved in ceramide metabolism were not found in this effort (i.e. dihydroceramide desaturase, ceramide-1P phosphatase). This is likely related to the fact that these genes have yet to be sequenced in the Pacific oyster. It is also possible that corresponding enzymes lack significant sequence homology. Once a complete genome is sequenced for this species, a more comprehensive analysis could be performed.

Sptlc1, responsible for accumulation of intracellular ceramide during cellular stress [5,22], is highly conserved in oysters including a 21 residue transmembrane region originally identified in *H. sapiens* [23]. The serine palmitoyltransferase identified in *Crassostrea gigas* has high homology with the LCB1 *Homo sapiens* isoform. There are two forms of *H. sapiens* Sptlc – LCB1 and LCB2 [23]. *H. sapiens* LCB2 has a conserved motif that binds pyridoxal phosphate (PLP) [23, 24], but LCB1 has an asparagine instead, which is homologous to the *C. gigas* sequence. In *H. sapiens*, LCB1 is necessary for the maintenance of LCB2 and does not perform the same catalytic functions [23]. More research is needed to determine if the functionality of specific Sptlc isoforms are conserved across taxa.

Crassostrea gigas 3KDSR shares conserved catalytic domains with all other amino acid sequences in the alignment, suggesting that its functionality is conserved across

taxonomic groups. 3-ketodihydrosphingosine reductase acts downstream of Sptlc. The product of the reaction catalyzed by Sptlc is 3-ketosphinganine. 3-ketosphinganine is reduced by a NADPH-dependent reductase to dihydrosphingosine. The enzyme that catalyzes this reaction is 3-ketodihydrosphingonsine reductase (3KDSR). 3KDSR contains two functional sites that are highly conserved, based on the amino acid alignment: an NADH/NADPH binding site and a catalytic site [18] (Figure 3). All four amino acid sequences – *C. gigas*, *Homo sapiens*, *Mus musculus*, and *Danio rerio* - share more of the catalytic site motif than previously described: *Tyr-Ser-X-Ser-Lys*, beginning at position 187 on the alignment [18] (Figure 3). The motif of the NADH/NADPH binding site is identical in its entirety – *Gly-Gly-Ser-Ser-Gly-Ile-Gly* – across all four sequences/taxa [18] (Figure 3).

The gene expression patterns observed for each gene are consistent with a role of ceramide metabolism in the stress response. The highest expression was observed in the gill tissue, which is rich with hemocytes, the primary immune cell in bivalves. Furthermore, exposure to *Vibrio vulnificus* significantly elevated expression of Cg-*AC*. There are several possible interpretations of this increase in gene expression that corroborate with the second messenger role of ceramide. For instance, increased expression of Cg-*AC* could indicate that ceramide is transformed into the lipid sphingosine, which then functions downstream as a signaling molecule in the *Crassostrea gigas* immune response. Sphingosine is an important signaling molecule in the vertebrate immune response and probably plays a similar role in invertebrates. Sphingosine is associated with the inhibition of the proliferation of Th2 T cells, inhibition of protein kinase C activity, regulation of the complement system, and inhibition of neutrophil respiratory burst [25, 26]. The roles that

300 sphingosine and other sphingolipids play in the immune response seem to be heavily
301 influenced by their concentrations [26], thus Cg-*AC* could be a pivotal enzyme regulating
302 levels of sphingosine in oyster.
303 An alternative explanation for the increased expression of Cg-*AC* during *Vibrio*
304 *vulnifcus* challenge suggests that ceramide is the primary signaling molecule in the
305 *Crassostrea gigas* immune response. An accumulation of ceramide in response to the *V.*
306 *vulnificus* exposure could have occurred and Cg-*AC* may be up-regulated to metabolize
307 ceramide after it has performed its signaling roles. Ceramide may have been produced to
308 increase signaling of immune pathways necessary for responding to bacterial exposure.
309 Increased expression of AC has been shown to decrease intracellular ceramide in mammals
310 [6, 4] and could very well play the same role in invertebrates. In support of this second
311 hypothesis, the genes Cg-*sptlc1* and Cg-*3KDSR,* responsible for the synthesis of ceramide,
312 showed trends towards up-regulation after *V. vulnificus* exposure, although the differences
313 in expression were not significant.
314 Here we report the identification of numerous genes in *Crassostrea gigas* involved in
315 the metabolism of ceramide, an important lipid signaling molecule. Gene expression
316 analysis suggests that ceramide is involved in the immune response of oysters exposed to
317 microbial pathogens. It should be noted that a limited number of genes were examined
318 here and targeted studies would be required to further elucidate the functional role of
319 ceramide metabolism in bivalves. For instance future efforts might directly quantify
320 sphingolipid levels and correlate levels with specific cellular function. Furthermore, it is not
321 known if lipid content in bivalve diets impacts stress physiology by influencing ceramide
322 levels. Characterizing how diet and other conditions affect ceramide metabolism could

323 offer a framework for better understanding mechanisms associated environmental effects
324 on immune function.
325
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337
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411

FIGURES

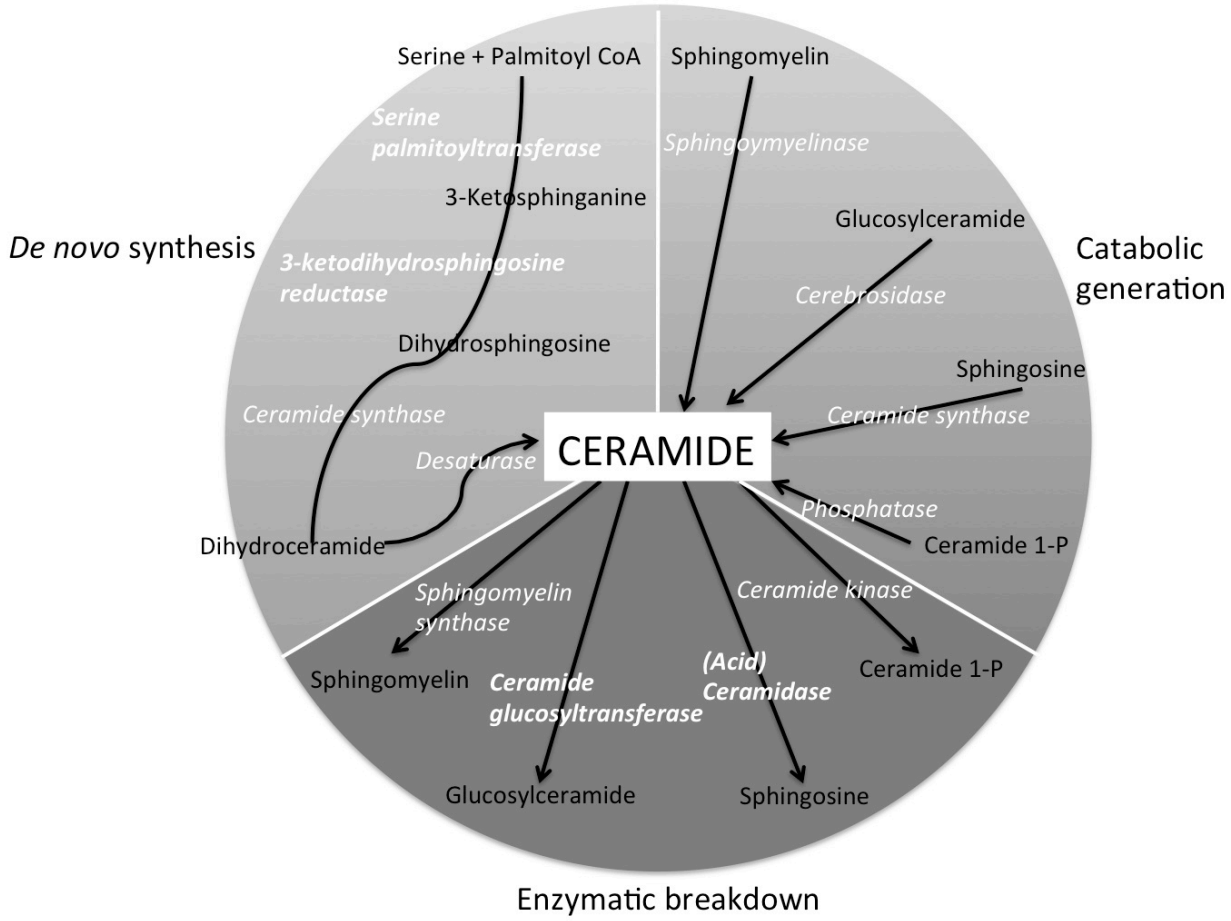


Fig. 1. Representation of the major players in the ceramide metabolism pathways. Enzymes are in white italics with the genes characterized as part of this study in bold: *serine palmitoyltransferase*, *3-ketodihydrosphingosine reductase*, *ceramide glucosyltransferase*, and *acid ceramidase*. Compounds that are either precursors, or the products of enzymatic break-down of ceramide, are shown in black. The pathway is adapted from [9 and 27].

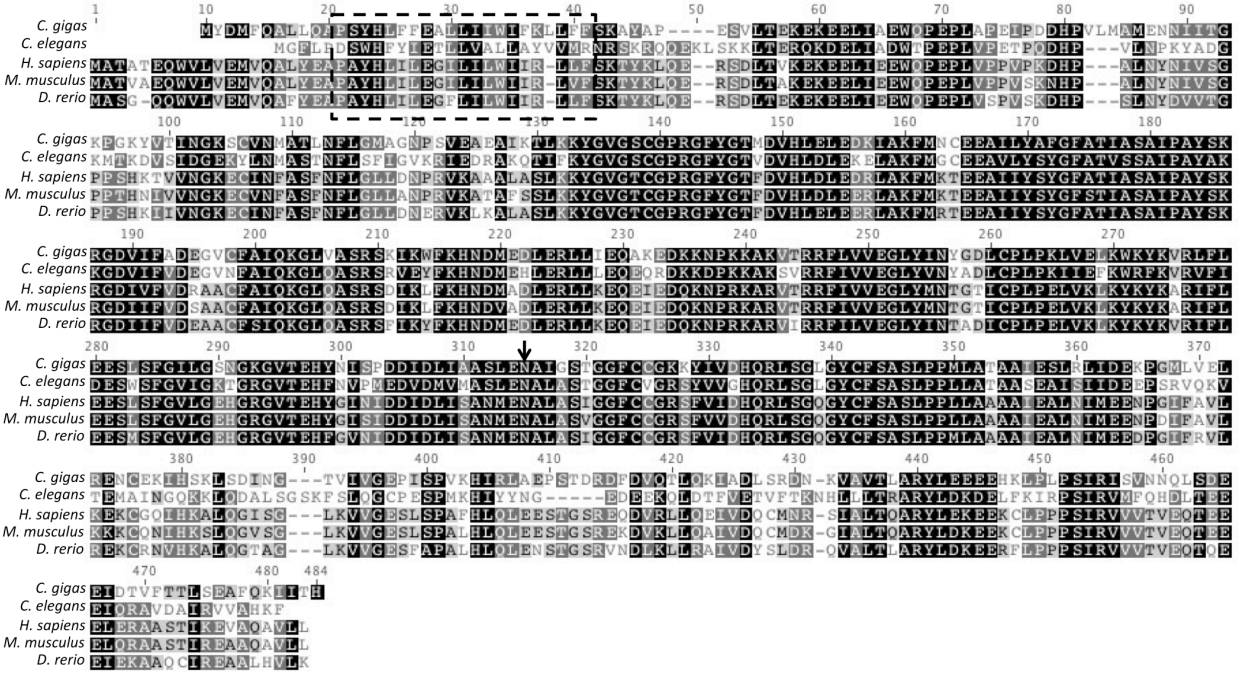


Figure 2. Amino acid alignment of translated Cg-*sptlc1* with protein sequence of *C. elegans* (GenBankAccession Number NP_001021978), *H. sapiens* (GenBank Accession Number NP_006406), *M. musculus* (GenBank Accession Number NP_033295), and *D. rerio* (GenBank Accession Number NP_001018307). Black shading indicates 100% similarity across sequences, dark gray is 80-100% similarity, light gray is 60-80% similarity, and white is less than 60% similarity. The transmembrane domain is marked by the dashed box and the asparagine that corresponds to the *H. sapiens* LCB1 isoform is marked with an arrow.

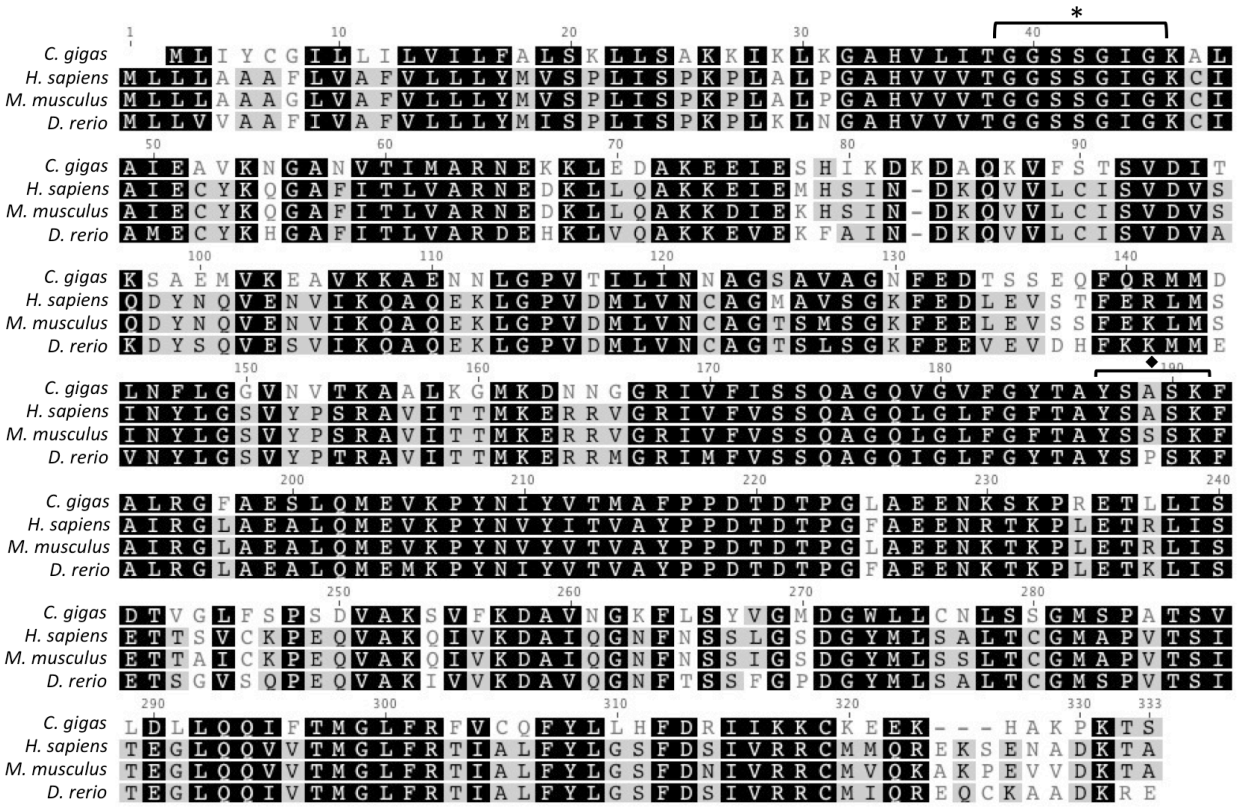


Figure 3. Amino acid alignment of translated Cg-*3KDSR* with protein sequence from *H. sapiens* (GenBank Accession Number Q06136), *M. musculus* (GenBank Accession Number NP_081810), and *D. rerio* (GenBank Accession Number NP_957433). Black shading indicates 100% similarity across sequences, dark gray is 80-100% similarity, light gray is 60-80% similarity, and white is less than 60% similarity. The conserved catalytic site is marked with an asterisk and the NADH/NADPH binding site and active site motif is marked with a diamond.

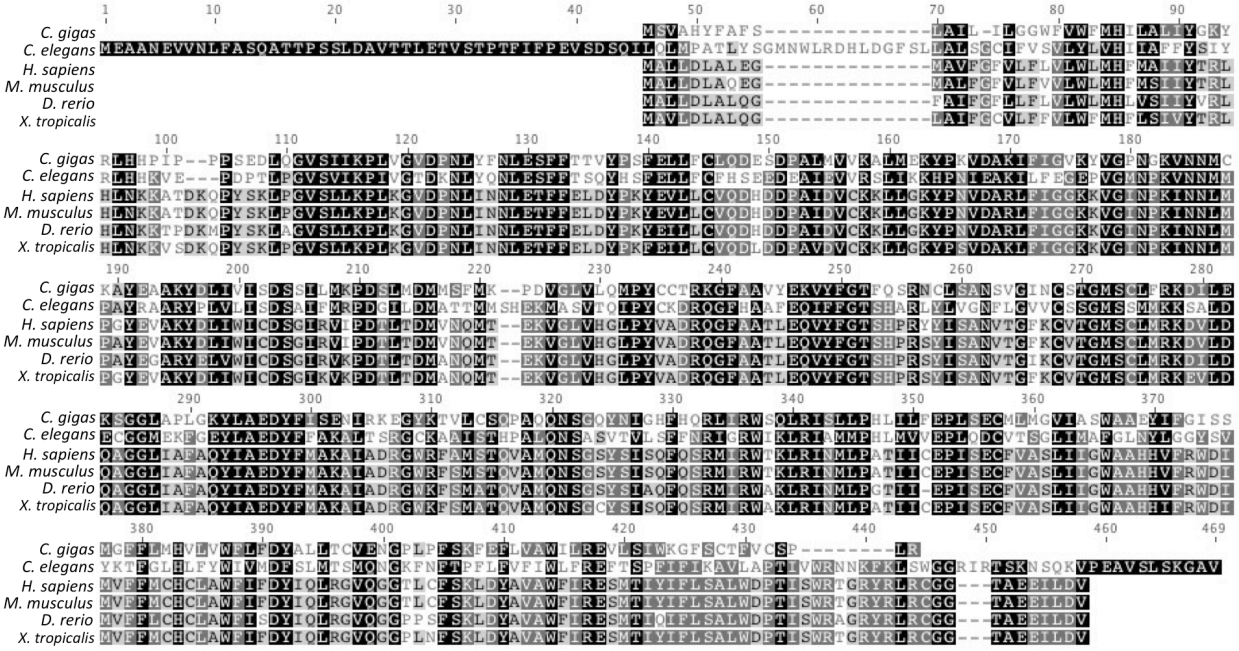
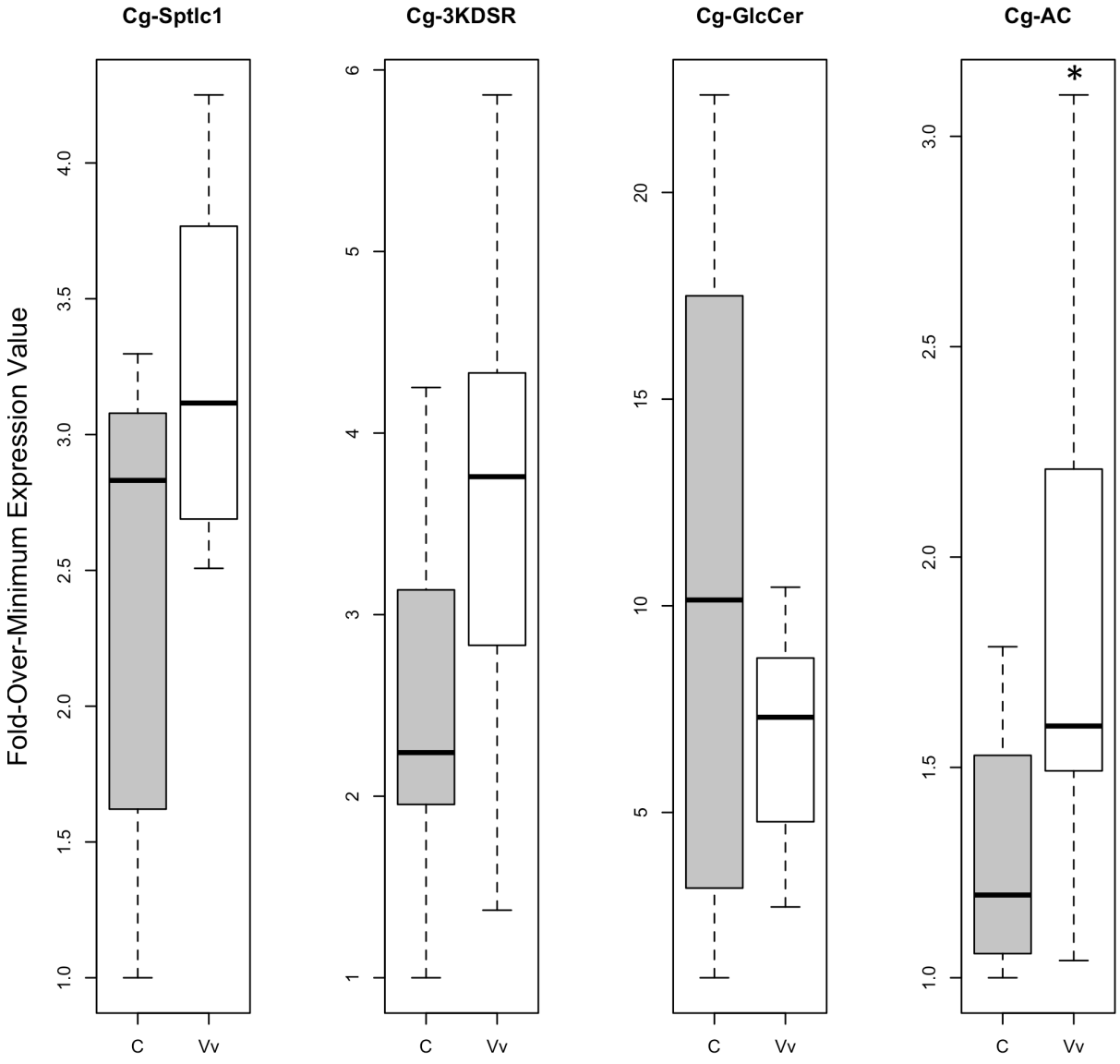


Figure 4. Amino acid alignment of translated Cg-*GlcCer* with ceramide glucosyltransferase protein sequences from *C. elegans* (GenBank Accession Number NP_506971), *H. sapiens* (GenBank Accession Number NP_003349), *M. musculus* (GenBank Accession Number NP_035803), and *X. tropicalis* (GenBank Accession Number Q5BL38). Black shading indicates 100% similarity across sequences, dark gray is 80-100% similarity, light gray is 60-80% similarity, and white is less than 60% similarity.

Figure 5. Amino acid alignment of translated Cg-*AC* with protein sequences from acid ceramidase in *C. elegans* (GenBank Accession Number NP_493173), *H. sapiens* (GenBank Accession Number NP_808592), *M. musculus* (GenBank Accession Number NP_062708), and *D. rerio* (GenBank Accession Number NP_001006088). Black shading indicates 100% similarity across sequences, dark gray is 80-100% similarity, light gray is 60-80% similarity, and white is less than 60% similarity.



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456 Figure 6. Expression values in gill tissue for *serine palmitoyltransferase-1* (Cg-*sptlc1*), *3-*
457 *ketodihydrosphingosine reductase* (Cg-*3KDSR*), *glucosylceramidase* (Cg-*GlcCer*), and *acid*
458 *ceramidase* (Cg-*AC*) . Gene expression values for the control ("C") oysters are represented
459 by the gray boxes, while the *V. vulnificus*-exposed ("Vv") oysters are represented with the
460 white boxes. Boxes represent the spread of the middle 50% of the data with the median
461 shown as the horizontal black line in the box. The dotted lines span the remaining data. An

asterisk indicates a significant difference in expression between exposed and control oysters.

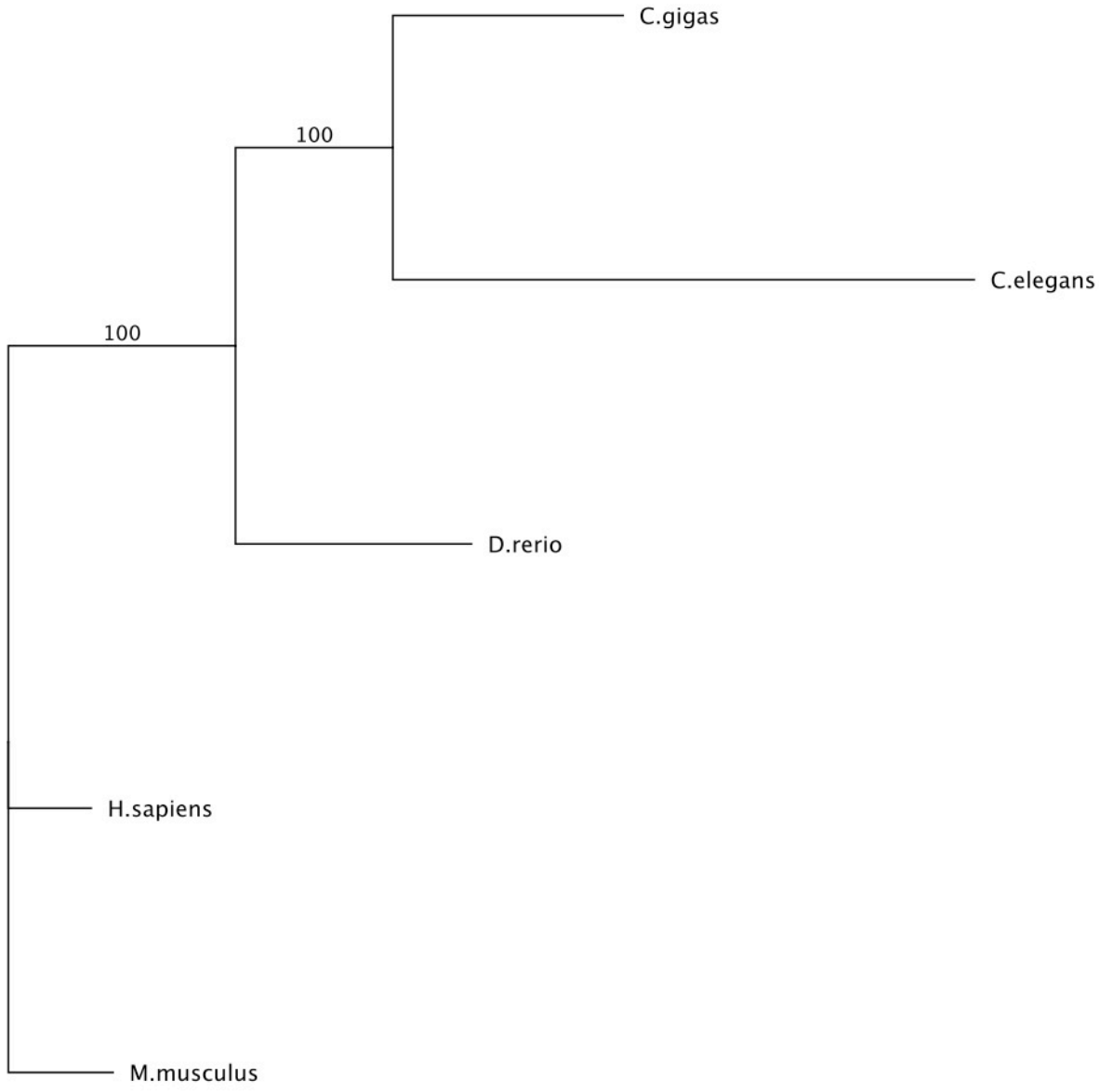

Figure 7. Maximum likelihood phylogenetic tree of the amino acid alignment of acid ceramidase in *C. gigas*, *C. elegans*, *H. sapiens*, *M. musculus*, and *D. rerio*. All other protein trees had similar topology to the one shown. The tree was created based on the James-Taylor-Thornton (JTT) model and bootstrapped 100 times.

470 TABLES

471 Table 1. Sequencing and qPCR primers for ceramide metabolism genes.

Gene	EF1α	Sptlc1	3KDSR
Description	Elongation factor 1 α	Serine palmitoyltransferase	3-ketodihydrosphingosine reductase
Sequencing Forward Primer	-	ATGGCGTCGACGTTCATTCC	AGCGACGGACCGAACTTACT
Sequencing Reverse Primer	-	CTGTTCCCCAATATTTCTGAC	TGTCTTGGGTTTTGCATCCTTC
Sequence Product Size (bp)	-	1483	1210
qPCR Forward Primer	AAGGAAGCTGCTGAGATGGG	TTCACAGCAAGCTGAGCGAT	GCAGTGCAGTGGCTGGAAAT
qPCR Reverse Primer	CAGCACAGTCAGCCTGTGAAGT	AAGTAGCGAGCCAACGTCAC	AGGCAGCCTTGGTGACATTG
qPCR Product Size (bp)	200	178	168

AC	Acid ceramidase	TGTGATTACACGATGTGGATACCG	CTGCTTCTGACTTCCGGTGT	1165	TGGACTCAAGTTGGCCAGGA	AAGGCTGGGGGAGAGTATCG	157
GlcCer	Glucosylceramide synthase	AGAGGCGAACACACGAAAGT	CCATATGGATAACACTTCTCG	1080	TTGGCCCAAACGGGAAAGTT	TGTCCATGAGCGAGTCTGGT	114

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473 Table 2. Genes associated with ceramide metabolism in *C. gigas* from the *in silico* search.

Gene ID	Gene Description	Species	e-value
Cg_4852	Serine palmitoyltrasnferase-1	*Pongo abelii*	0
Cg_877	Acid ceramidase	*Rattus norvegicus*	2E-146
Cg_14141	3-ketodihydrosphingosine reductase	*Mus musculus*	5e-46
Cg_29918	Ceramide glucosyltransferase	*Xenopus tropicalis*	9e-122
Cg_21728	Acid sphingomyelinase	*Mus musculus*	5e-46

Cg_16356	Ceramide kinase	*Homo sapiens*	1e-27
Cg_16726	Cerebrosidase	*Mus musculus*	5e-139
Cg_17230	Neutral ceramidase	*Oryza sativa*	5e-96
Cg_1560	Caspase 7	*Homo sapiens*	4e-10
Cg_23531	Caspase 8	*Homo sapiens*	8e-54
Cg_252	TNF receptor-associated factor 2	*Mus musculus*	1e-53
Cg_3248	TNF receptor-associated factor 3	*Mus musculus*	2e-30
Cg_31180	TNF receptor-associated factor 4	*Homo sapiens*	4e-39
Cg_6808	Neutral sphingomyelinase	*Caenorhabditis elegans*	4e-10
Cg_20643	Dihydrosphingosine 1-phosphate phosphatase	*Schizosaccharomyces pombe*	3e-9
Cg_26221	Sphingosine-1-phosphate phosphatase	*Mus musculus*	8e-20
Cg_7888	Sphingosine-1-phosphate lyase	*Dictyostelium discoideum*	6e-9
HS213433	Sphingomyelin synthase	*Homo sapiens*	1e-100
HS185280	Ceramide synthase	*Mus musculus*	5e-83
HQ425701	Inhibitor of apoptosis	*Crassostrea gigas*	-

HQ425703	Caspase 1	*Crassostrea gigas*	-
HQ425705	Caspase 2	*Crassostrea gigas*	-
HQ425699	Fas-associated receptor with Death Domain	*Crassostrea gigas*	-

Table 2 Legend. Genes associate with ceramide metabolism were identified in *C. gigas* by searching of publicly available databases. Sequences assembled from short read archive data and expressed sequence tags were given a Gene ID code that corresponds to the sequence in the supplementary information (S1). For each of these contiguous sequences, the top BLASTx hit description, corresponding species, and e-value are provided. Two genes that were identified as a single EST (sphingomyelin synthase and ceramide synthase) are denoted with their respective GenBank Accession Numbers. An additional four genes (GenBank Accession Numbers HQ425699, HQ425701, HQ425703, and HQ425705) have been previously characterized in *C. gigas* [20].