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Transcriptomic responses of *Artemia salina* exposed to an environmentally relevant dose of *Alexandrium minutum* cells or Gonyautoxin2/3

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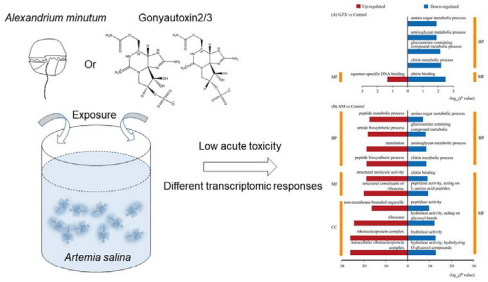
HIGHLIGHTS

- *A. salina* was not very sensitive to *A. minutum* or GTX2/3 exposure.
- Exposure to *A. minutum* or GTX2/3 induced HSP70 expression in *A. salina*.
- Transcriptional responses of *A. salina* to *A. minutum* or GTX2/3 were different.
- Induced ribonucleoprotein expression protected *A. salina* from exposure to *A. minutum*.
- Exposure to GTX2/3 inhibited formation of chitin in *A. salina*.

GRAPHICAL ABSTRACT



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ABSTRACT

Toxicities of the marine algae *Alexandrium minutum* and its excreted gonyautoxins (GTXs) to the marine crustacean *Artemia salina* were investigated. Mortality was observed for neither larvae nor adult *A. salina* exposed to *A. minutum* at a density of 5000 cells/mL or 0.5 μM GTX2/3. After exposure, the full transcriptome of adult *A. salina* was assembled and functionally annotated. A total of 599,286 transcripts were obtained, which were clustered into 515,196 unigenes. Results of the transcriptional effect level index revealed that direct exposure to the toxic algae *A. minutum* caused greater alterations in the transcriptome than did exposure to the extracellular product GTX2/3. Mechanisms of effects were different between exposure of *A. salina* to *A. minutum* cells or GTX2/3. Exposure to *A. minutum* modulated formation of the ribonucleoprotein complex and metabolism of amino acids and lipids in *A. salina*. Exposure to GTX2/3 exposure inhibited expression of genes related to metabolism of chitin, which might result in disruption of molting process or disturbed sheath morphogenesis. Overall, effects on transcription observed in this study represent the first report based on application of next generation

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Microalgae
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sequencing techniques to investigate the transcriptomic response of *A. salina* exposed to an environmentally realistic level of *A. minutum* or GTX2/3.

1. Introduction

Proliferation of toxic microalgae has become a global threat to marine environments, human health and aquaculture (Brecely and Shumway, 1998). The genus *Alexandrium* is among the most common alga that produces blooms and is responsible for paralytic shellfish poisoning (PSP), which is one of the most critical and widespread harmful microalgae poisoning syndromes (Etheridge, 2010). There are a number of species in the genus *Alexandrium*, among which *Alexandrium minutum* is the most widespread in coastal areas all over the world. For example, *A. minutum* at population densities, ranging from a few hundred cells/mL to more than a million cells/L have been reported in Europe, USA, Australia and Asian countries (Anderson et al., 2012; Bravo et al., 2008). The greatest population density of *A. minutum* was 1.4×10^8 cells/L, which was observed in the inner basin of the Cape Town harbor from November 2003 to February 2004 (Pitcher et al., 2007).

A. minutum produces primarily gonyautoxins (GTXs): GTX1, GTX2, GTX3 and GTX4 (Hallegraeff et al., 1991; Hwang and Lu, 2000). Among the four isomers of GTX, GTX2 and GTX3 are the two main components produced by *A. minutum*, and the molar ratio of GTX2 and GTX3 among the GTXs that excreted by *A. minutum* samples near Taiwan was as great as 75.42% (Wu et al., 2005). GTXs are transferred through the food chain and accumulated to greater concentrations in marine organisms, especially in filter-feeding bivalves (Burrell et al., 2016). For example, in the nobel scallops (*Chlamys nobilis*) collected from coastal areas of Hong Kong, the mean, total concentration of GTXs was 320 μg saxitoxin equivalents (STXeq)/100 g soft tissue (Zhou et al., 1999). Humans can become intoxicated after ingestion of seafood contaminated with GTXs. There has been one clinical case that ingestion of contaminated *Rutitapes philippinarum* caused paralytic illness, cardiovascular shock and respiratory arrest and eventually led to death of patients (Lin et al., 2009). As a group of neurotoxic compounds, toxicity of GTXs or *A. minutum* cells have been mainly investigated using mammals (Selwood et al., 2016), human cells (Alonso et al., 2016) and small mammals (Andrinolo et al., 2002a,b). In addition, Jiang et al. (2010) studied adverse effects of *A. minutum* to early stages of the fish *Sparus microcephalus*. However, the effect of GTXs or *A. minutum* on species of lower trophic levels, in particular invertebrates and specifically crustaceans was largely unknown.

Zooplankton plays an important role in ecosystems processes since they transfer energy from primary producers to consumers of higher trophic levels. Artemia are ideal prey of juvenile fishes in both natural aquatic environments and aquaculture systems. However, to the best of our knowledge, few studies have evaluated effects of *A. minutum* to marine zooplankton. *Artemia salina* is widely distributed in salt lakes and coastal areas around the world, and plays an important role in energy flow of these saltwater food chains (Nunes et al., 2006). A number of physiological characteristics, such as rapid growth, short breeding cycle, ready availability and strong adaptability of culture in laboratory, make *A. salina* a suitable model species for toxicological studies (Barahona and Sánchez-Fortún, 1996; Libralato et al., 2016). In recent years, *A. salina* has been adopted specifically to investigate effects of blooms of hazardous algae (Botes et al., 2003; Giussani et al., 2015).

High-throughput transcriptomic analysis, for example RNA-seq analysis based on Illumina/Solexa or Applied bio-systems SOLiD sequencing technology, can help identify potential biomarker genes and unravel underlying molecular mechanisms (Martin and Wang, 2011; Mu et al., 2015). In recent years, digital gene expression (DGE) analysis based on these high throughput sequencing platforms have been applied to study multiple aquatic organisms such as yesso scallop (*Patinopecten yessoensis*), Japanese medaka (*Oryzias latipes*), Pacific oyster (*Crassostrea gigas*) and Farrer's scallop (*Chlamys farreri*) (Fu et al., 2014; Oh et al., 2012; Wang et al., 2013; Zhao et al., 2012).

In the present study, *A. salina* was used as the study organism to investigate effects of exposure to *A. minutum* or the extracellular product GTX2/3. Transcriptomic profiles of *A. salina* upon exposure to environmentally relevant densities of *A. minutum* or concentrations of GTX2/3 were investigated by use of next-generation sequencing to identify genetic networks and pathways responding to and modulated by exposure to *A. minutum* or GTX2/3.

2. Materials and methods

2.1. Cultures of algae and Artemia

A. minutum were originally collected from Daya Bay, China and identified as *A. minutum* Halim (AMTK4). The algae were cultured in f/2 medium using filtered artificial seawater (FASW; 30 ± 1 ppt, 0.45 μm filter membrane), and the cultures were maintained at 20 ± 1 °C under a light/dark cycle of 14 h/10 h for more than a year.

Larvae of *A. salina* (<24 h) were obtained from hatching cysts, which were commercially available (YEE, Tianjin, China). In brief, approximately 1 g of dehydrated cysts were incubated in FASW at 25 °C for 24 h (light/dark cycle of 14 h/10 h), and hatched larvae were separated with mesh net (pore size of 50 μm) and transferred to a 100 mL beaker with FASW before subjected to toxicity test. Adult animals were obtained by culturing the hatched larvae for 14 days in three small aquariums with 4 L FASW (at the same conditions as for the algae culture). *Artemia* were fed green algae *Chlorella* sp. at a density of approximate 10^6 cells/mL once every day.

2.2. Acute toxicity test of A. minutum or GTX2/3 exposure

Static 24 h acute toxicity test for *A. minutum* (at its stationary phase of growth) or GTX2/3 (purity > 97%, Cifga Laboratory, Spain) was conducted for both larvae and adult *A. salina* with the same exposure strategy except the volume of test solutions (4 mL for larvae and 50 mL for adults; ten individuals in each replicate and four biological replicates for each treatment). Test solutions at different designated concentrations (0.1, 0.2 and 0.5 μM) of GTX2/3 were prepared by diluting the standard solutions with FASW. The number of *A. minutum* cells in cultures were counted and algal cells were then diluted with FASW to the designated test densities (10^3, 2.5×10^3, 5×10^3 and 10^4 cells/mL). Acute exposures were all conducted at 25 °C under a light/dark cycle of 14 h/10 h.

2.3. PSP analysis

Identification and quantification of PSP, including GTX1, GTX2,

GTX3, GTX4, GTX5, saxitoxin (STX), neosaxitoxin (neoSTX), dcsaxitoxin (dcSTX), dcgonyautoxin 2 (dcGTX2), dcgonyautoxin3 (dcGTX3), sulfocarbamoyl toxin 1 (C1) and sulfocarbamoyl toxin 2 (C2) in exposure solutions from *A. minutum* exposure group (AM) followed the national standard method (GB 5009.213−2016) with slight modifications. Briefly, the algal cultures (5.0 mL) were filtered through 0.22 μm filter membrane. The filtrate was collected in a 10 mL centrifuge tube. Then the filtrate was filtered through 0.22 μm filter membrane again and 10 μL of the samples were subjected directly to analysis with liquid chromatography − tandem mass spectrophotometry (LC-MS/MS). A high performance liquid chromatography system (Dionex, CA, USA) was coupled online to a triple quadrupole mass spectrometer (API4000 AB SCIEX, MA, USA). Chromatographic separation was carried out with an Ultimate® HILIC Amide column (5 μm, 4.6 × 250 mm; maintained at 30 °C; Welch, China) and a multiple reaction monitoring (MRM) scan was performed to detect GTXs. During the analyses, the electrospray ionization (ESI) parameters were set as follows: ion spray voltage, 4.5 kV; capillary temperature, 300 °C; and ion spray gas1: 50 L/min. The detailed analysis parameters for LC-MS/MS were listed in supplementary materials (Table S1). For the sake of comparison, concentrations of GTX were all transformed to STXeq, which was used in results and discussion sections thereafter in this study. STXeq were calculated based on toxicity equivalency factors (TEFs) set by the European Food Safety Authority (EFSA) (2009), which for STX-group toxins are 1.0 for GTX1, 0.4 for GTX2, 0.6 for GTX3, and 0.7 for GTX4 (Equation (1)).

$$\mathrm{STXeq} = 1.0 \times \mathrm{Conc.}_{(\mathrm{GTX1})} + 0.4 \times \mathrm{Conc.}_{(\mathrm{GTX2})} + 0.6 \times \mathrm{Conc.}_{(\mathrm{GTX3})} + 0.7 \times \mathrm{Conc.}_{(\mathrm{GTX4})} \quad (1)$$

where Conc. is the measured concentration of a specific gonyautoxin congener in exposure solutions from *Alexandrium minutum* exposure.

2.4. RNA extraction, gene library preparation, and sequencing

To obtain a global view of the effects of *A. minutum* or GTX2/3 exposure, *A. salina* adults were randomly collected from the culture tank and exposed to 0.5 μM of GTX2/3 or 10^4 cells/mL of *A. minutum* for 24 h. There were three replicates (with about 200 adults randomly collected from the culture tanks in each replicate) for each treatment (i.e., GTX2/3 exposure, *A. minutum* exposure and FASW exposure, which served as the unexposed control group). The exposure strategy was the same as that used for the acute test. After exposure, animals were collected with a mesh net (pore size: about 100 μm) and transferred to 1.5 mL centrifuge tubes. Collected samples were homogenized with a pestle and total RNA was isolated with Trizol Reagent (Invitrogen Life Technologies). RNA quality, integrity, and concentration were determined by using a NanoDrop spectrophotometer (Thermo Scientific) and only samples with a 260/280 nm ratio of 1.8−2.0 were analyzed farther. Integrity of RNA was verified with 1.5% agarose−formaldehyde gel. Three micrograms of RNA were used as input material for the RNA sample preparations. Due to contamination of RNA in one sample from *A. salina* exposed to *A. minutum*, a total of 8 sequence libraries were constructed, including three libraries from control groups, three libraries from *A. salina* exposed to GTX2/3 and two libraries from *A. salina* exposed to *A. minutum*. Libraries of sequences were generated using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in a proprietary fragmentation buffer obtained from Illumina. First strand cDNA was synthesized using random oligonucleotides and SuperScript II. Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities then enzymes were removed. After adenylation of the 3' ends of the DNA fragments, Illumina PE adapter oligonucleotides were ligated to prepare for hybridization. To select cDNA fragments of the preferred 200 bp in length, the library fragments were purified using the AMPure XP system (Beckman Coulter, Beverly, CA, USA). DNA fragments with ligated adaptor molecules on both ends were selectively enriched using Illumina PCR Primer Cocktail in a 15 cycle PCR reaction. Products were purified (AMPure XP system) and quantified using the Agilent high sensitivity DNA assay on a Bioanalyzer 2100 system (Agilent). The library was then sequenced on a Hiseq platform (Illumina) by Shanghai Personal Biotechnology Cp. Ltd.

2.5. De novo assembly and gene function annotation

Raw data in FASTQ format were first processed by Cutadapt (version 1.15) before de novo assembly. Clean reads were obtained by removing reads with an adaptor, low quality reads (<Q20) and reads with length less than 50 bp. Meanwhile, Q20, Q30, GC content, and N (%) of the clean reads were calculated. Then, high-quality, clean reads were assembled using Trinity software (r20140717, K-mer 25 bp) to construct transcript and unigene sequences. Afterwards, assembled unigenes were annotated for function against several public databases, i.e., NCBI non-redundant protein sequences (NR) database, Gene Ontology (GO) database, Kyoto Encyclopedia of Genes and Genome (KEGG) database, evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) database and Swiss-Prot database with a threshold E-value $\leq 1e^{-5}$ (Conesa et al., 2005).

2.6. Differentially expresssed genes and enrichment analysis

Clean reads were mapped to each assembled unigene by RSEM software. The transcript abundances were measured as Fragments per kilobase of transcript sequence per millions base pairs sequenced (FPKM). Analysis of differentially expressed genes (DEGs) was performed by use of DESeq software (version 1.18.0). For better accuracy, we focused on unigenes ≥300 bp (a number of 259,573 unigenes). Criteria for DEGs were (1) P value < 0.05 and (2) fold change (FC) > 2. GO and KEGG pathway enrichment analyses for DEGs were conducted with GO:TermFinder and KEGG Automatic Annotation Server (http://www.genome.jp/tools/kaas/), respectively. Up-regulated and down-regulated DEGs were analyzed separately. For GO enrichment, the corrected P value < 0.05 was selected as the threshold for significant enrichment, while for KEGG enrichment, P value < 0.05 and false discovery rate (FDR) < 0.05 were adopted as criteria for significant enrichment.

2.7. Validation of transcriptomics data

Seven genes (i.e., 3 genes for the GTX exposure group and 4 genes for the *A. minutum* (AM) exposure group) were selected for confirmation of transcriptomics data by using quantitative real time PCR (qRT-PCR). Primer sequences were listed in Table S2. Sequences of these selected unigenes were compared with the homologues (blastx) to verify our annotation. RNA samples of *A. salina* were extracted with the same method as described above, and concentrations of RNA were determined with Nanodrop 2000 (Thermo Scientific, USA). Two micrograms of isolated RNA was applied for complimentary DNA (cDNA) synthesis with a Universal

RT-PCR Kit (Solarbio, China), and 2 μL of cDNA templates were applied in each reaction. The RT-PCR were carried out on a 7500 RT-PCR systems (Applied Biosystems, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was adopted as the reference gene. Reaction conditions were: 95 °C/4 min, 40 cycles of 95 °C/15 s, 60 °C/30 s and 72 °C/30 s. Melting curves were determined with: 5 °C/1 min, and 80 cycles of 65 °C/5 s with 0.5 °C increase per cyle. Relative expression of the target genes were calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.8. Statistical analysis

Three biological replicates were conducted for each qRT-PCR experiment, and significant expression from control after GTX2/3 or *A. minutum* exposure were compared by student's *t*-test after assuring that the assumptions of normality (one sample Kolmogorov-Smirnov test) and variance homogeneity (Levene test) had been met. In addition, to assess overall alteration of transcriptome of *A. salina* after GTX2/3 or *A. minutum* exposure, transcriptional effect level index (TELI) was calculated. TELI converts the information-rich toxicogenomic data into an integrated endpoint, which can reflect the overall transcriptional alteration (Gou and Gu, 2011). Detailed calculation methods are shown in the Supplementary material (Determination of TELI). As for TELI comparison, because the sample size of AM group was two, the values of control group and GTX exposure group were compared with the average values of the two samples for AM treated group, and the significant difference was compared with one sample *t*-test. Differences between TELI values of control and GTX group was compared by student's *t*-test. Differences were considered significant at $0.01 < P < 0.05$ and highly significant when $P < 0.01$. Statistical analyses were conducted by use of SPSS 23.0 (SPSS Inc., USA). Partial least squares-discriminant analysis (PLS-DA) analysis was carried out by caret packages within R.

3. Results

3.1. Acute toxicity of AM or GTX2/3 to A. salina

Acute exposures of both larvae and adults of *A. salina* were conducted for GTX2/3 or *A. minutum*. As for GTX2/3, no mortality of larvae or adult *A. salina* was observed even at 0.5 μM. As for *A. minutum*, no lethality was observed in adult *A. salina* after exposure to all densities of algal cells. A mean mortality of 13.3% was observed for larvae of *A. salina* after exposure to 10^4 cells/mL of *A. minutum*, while no lethality was observed at lesser densities (i.e., 10^3, 2.5×10^3 or 5.0×10^3 cells/mL).

LC-MS/MS analysis revealed that only GTX1/4 and GTX2/3 were detected in *A. minutum* exposure solutions, while other toxins, including GTX5, STX, neoSTX, dcSTX, dcGTX2, dcGTX3, C1 and C2, were not detected. Actual concentrations of GTX congeners in exposure solutions from 10^4 cells/mL of *A. minutum* group were 11.88 ± 1.6 ng/mL for GTX1, 83.5 ± 3.5 ng/mL for GTX2, 42.23 ± 2.4 ng/mL for GTX3, and 2.46 ± 0.7 ng/mL for GTX4. The STXeq for 10^4 cells/mL of the *A. minutum* group was 72.34 ± 3.2 ng/mL (Table S3) based on equivalency factors defined by the European Food Safety Authority (2008). The nominal STXeq for the 0.5 μM GTX2/3 group was 90.16 ng/mL.

3.2. RNA-seq and de novo assembly

Transcriptomic analysis of adult *A. salina* exposed to 0.5 μM GTX2/3 or 10^4 cells/mL of *A. minutum* for 24 h were conducted by use of Next-Generation Sequencing/Illumina technology. Eight *A. salina* DEG libraries were sequenced for individuals exposed for 24 h to control (C) and two experimental groups (GTX2/3 and *A. minutum* exposure). A total of 40,184,250 to 45,808,962 raw reads were generated in these eight libraries (Table S4). After removing lesser quality reads, the number of clean reads ranged from 39,917,110 to 45,521,926. Values of the Q20, Q30 percentage and GC percentage (Table S4) indicated reliabilities of libraries. Based on use of Trinity software, a total of 599,286 transcripts were obtained, which were clustered into 515,196 unigenes. The raw data were deposited at NCBI SRA under the accession number: PRJNA545769.

3.3. Gene functional annotation

Functional gene annotation against NR, GO, KEGG, eggNOG, and SwissProt databases with a cut-off E-value $< 10^{-5}$ were performed to get insight into comprehensive information of these genes. As a result, a total of 44.06% of unigenes was successfully annotated, and 0.56% of unigenes were annotated in all database (Table S5). The E-value distribution showed that matches with an E-value of 1×10^{-15} to 1×10^{-5} had the largest ratio (44.7%; Fig. 1A). As shown

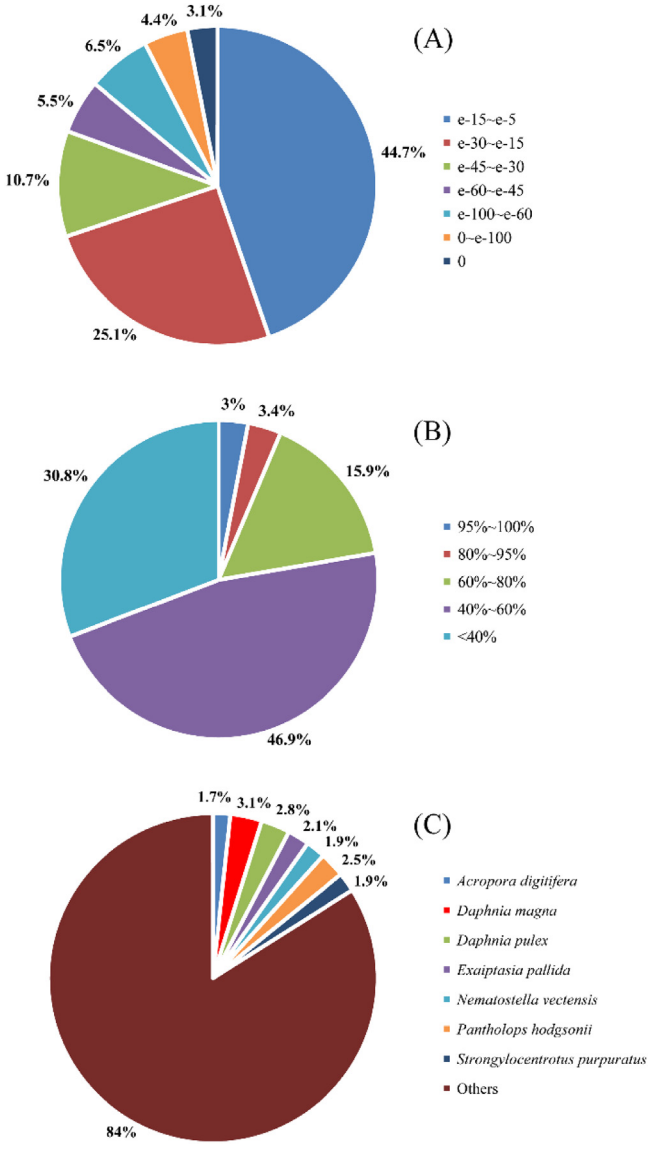

Fig. 1. Alignment statistics of the transcriptome against NR databases. **(a)** E-value distribution; **(b)** similarity distribution; and **(c)** species distribution.

in Fig. 1B, about 6.4% of unigenes had a strong similarity greater than 80% to available animal sequences, whereas 62.8% of unigenes had a similarity value between 40% and 80%. The species distribution analysis showed that approximately 3.6% of unigenes was similar to that of planktonic crustacean *Daphnia magna* (3.6%; Fig. 1C), followed by *Daphnia pulex* (3.3%), *Pantholops hodgsonii* (2.9%), *Exaiptasia pallida* (2.5%), *Nematostella vectensis* (2.2%), and *Strongylocentrotus purpuratus* (2.2%). These results demonstrated both the close phylogenetic relationship between *A. salina* and these matched species and also abundant genomic information for these matched species.

GO annotation revealed that 142,045 unigenes (27.57%) significantly matched in GO database (Fig. 2). Unigenes were annotated to three main GO categories, which were biological process, cellular component and molecular function. Within the biological process category, 23.9% of unigenes were assigned to cellular process, 20.9% to metabolic process, and 17.3% to single-organism process. Within the cellular component category, the majority of unigenes were assigned to cell (20.6%), cell part (20.5%) and organelle (14.6%) terms. Moreover, the terms catalytic activity and binding were the main categories of molecular function.

For KEGG annotation, 4860 (0.94%) unigenes were annotated for *A. salina* transcriptome against the KEGG database (Fig. 3). All annotated unigenes were assigned to five main categories, including metabolism (1367 unigenes), genetic information processing (1075 unigenes), environmental information processing (602 unigenes), cellular processes (706 unigenes) and organismal systems (1110 unigenes). Among them, signal transduction and translation were the most represented KEGG pathways in *A. salina* transcriptome.

3.4. Global transcripomics response of *A. salina* to *A. minutum* or GTX2/3

PLS-DA analysis was performed for all unigenes of *A. salina* after exposure. The PLS-DA score plot showed a clear separation between all three groups along the component 2 axis (Fig. 4A). These results demonstrated that exposure to either *A. minutum* or GTX2/3 caused significant changes in transcriptomics of *A. salina*. In addition, those exposed to *A. minutum* were also clearly separated from *A. salina* exposed to GTX2/3, which indicated dissimilar patterns of expression of genes in *A. salina* after exposure to *A. minutum* or GTX2/3. To quantify and compare overall transcriptomic responses of *A. salina* exposed to *A. minutum* or GTX2/3, TELI values were calculated (Fig. 4B). TELI values of *A. salina* exposed to either *A. minutum* or GTX2/3 were significantly greater than that of the control group. In addition, the TELI value of *A. salina* exposed to *A. minutum* was also greater than that of *A. salina* exposed to GTX2/3.

3.5. Identification of DEGs

Profiles of expression of DEGs were determined to identify differences in expression of genes between the control and two experimental groups. For better accuracy, only those unigenes $\geq$300 bp (a total of 259,573 genes) were analyzed. Compared to the control group, 20,189 and 254 unigenes were identified as DEGs for groups of *A. salina* exposed to either *A. minutum* or GTX2/3, which met the criteria of P value < 0.05 and $|\text{fold change (FC)}| > 2$. For *A. salina* exposed to *A. minutum*, 19,301 unigenes were up-regulated and 888 genes were down-regulated, while only 108 and 146 genes were up- and down-regulated in *A. salina* exposed to GTX2/3 (Fig. 4C). Among these DEGs, only 40 up-regulated and 79 down-regulated unigenes were communally altered for both treatment groups (Fig. 4C).

DEGs of the control group were subjected to GO analysis to identify potentially affected functions of *A. salina* by either *A. minutum* or GTX2/3 exposure. Compared to the control group, significantly up-regulated GO terms in the GTX group were found for sequence-specific DNA binding belonging to molecular functions. In addition, down-regulated GO terms in the GTX group included chitin binding in molecular function ontology, and chitin metabolic process, glucosamine-containing compound metabolic process, aminoglycan metabolic process and amino sugar metabolic process in biological process ontology (Fig. 5A). In *A. salina* exposed to *A. minutum*, 54 functional terms were determined to be up-regulated, among which, 8, 23 and 23 of the terms were classified to molecular functions, cellular component and biological process, respectively (**Supplemental material,** Table S6**).**

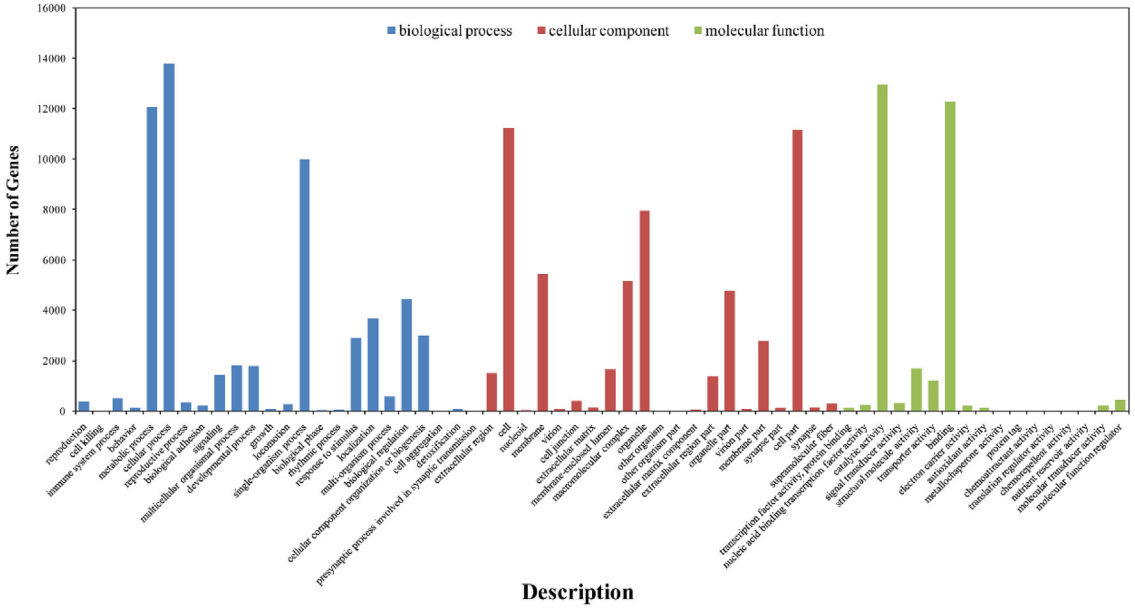

Fig. 2. GO terms distribution of annotated unigenes in *Artemia salina*.

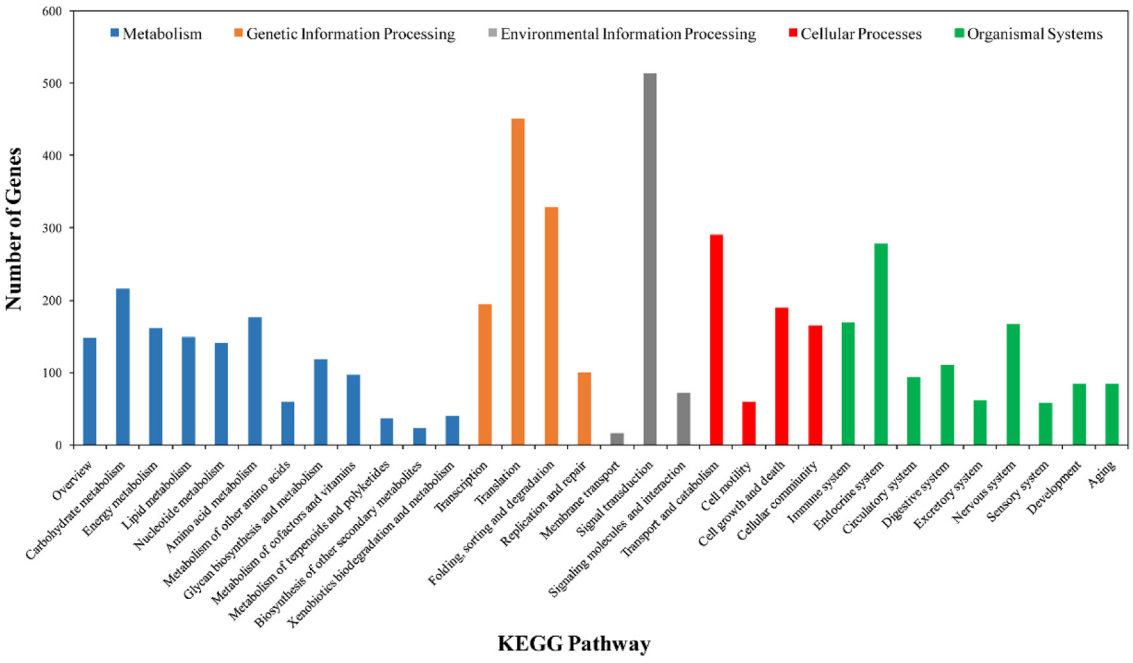

Fig. 3. KEGG classification of *Artemia salina* transcriptome into functional groups.

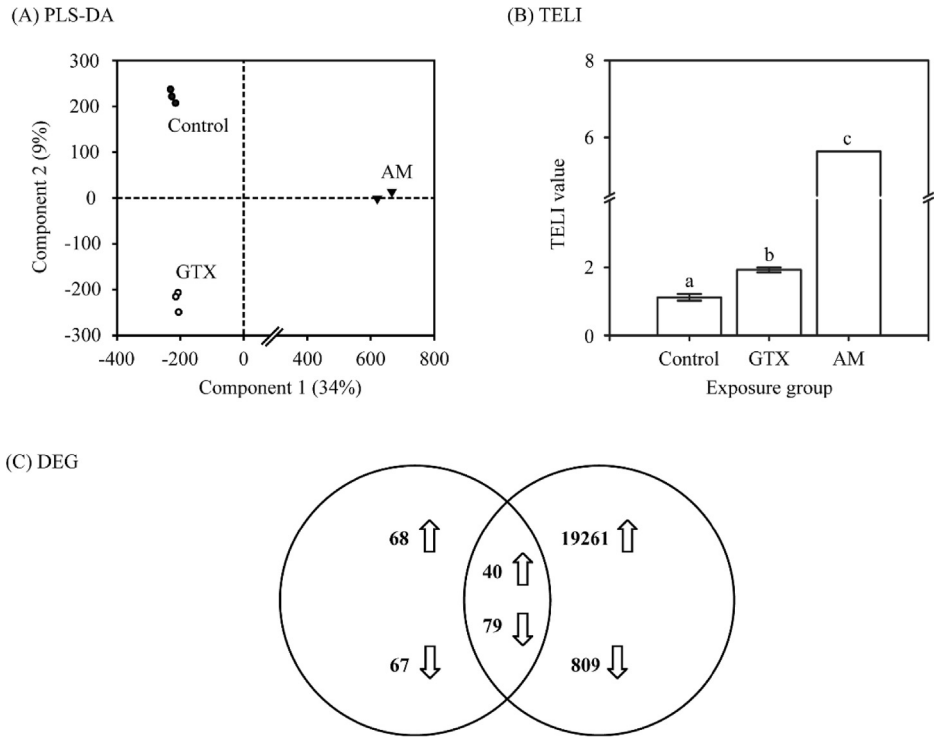

Fig. 4. **(A)** Partial least squares discriminant analysis (PLS-DA) analysis for global transcriptomic database. **(B)** Transcriptional effect level index (TELI) of the *Artemia salina* after exposure to *Alexandrium minutum* (AM) or Gonyautoxin2/3 (GTX). Values with different letters denote statistically significant differences. **(C)** Numbers of differentially expressed genes (DEGs) induced by GTX2/3 (GTX) or *Alexandrium minutum* (AM) exposure. C: control group.

Sgnificantly down-regulated enriched GO terms in *A. salina* exposed to *A. minutum* included 27 molecular function terms, 4 cellular component terms, and 14 biological process terms (**Supplemental material,** Table S6**)**. The 10 most significantly enriched GO terms for both up-regulation and down-regulation (according to the ranking of *P* values) are presented (Fig. 5B**)**.

KEGG pathway enrichment was also conducted for all DEGs. Relative to the control group, only two pathways were down-regulated, i.e. longevity regulating pathway and protein processing in endoplasmic reticulum in individuals exposed to GTX2/3. No pathways were found to be significantly up-regulated after exposure to GTX2/3 (**Supplemental material,** Table S7). As for individuals exposed to *A. minutum*, significantly enriched pathways are shown (Fig. 6**)**. Fatty acid biosynthesis, fatty acid metabolism, glycine, serine, and threonine metabolism, methane metabolism and spliceosome were the five most up-upregulated pathways in

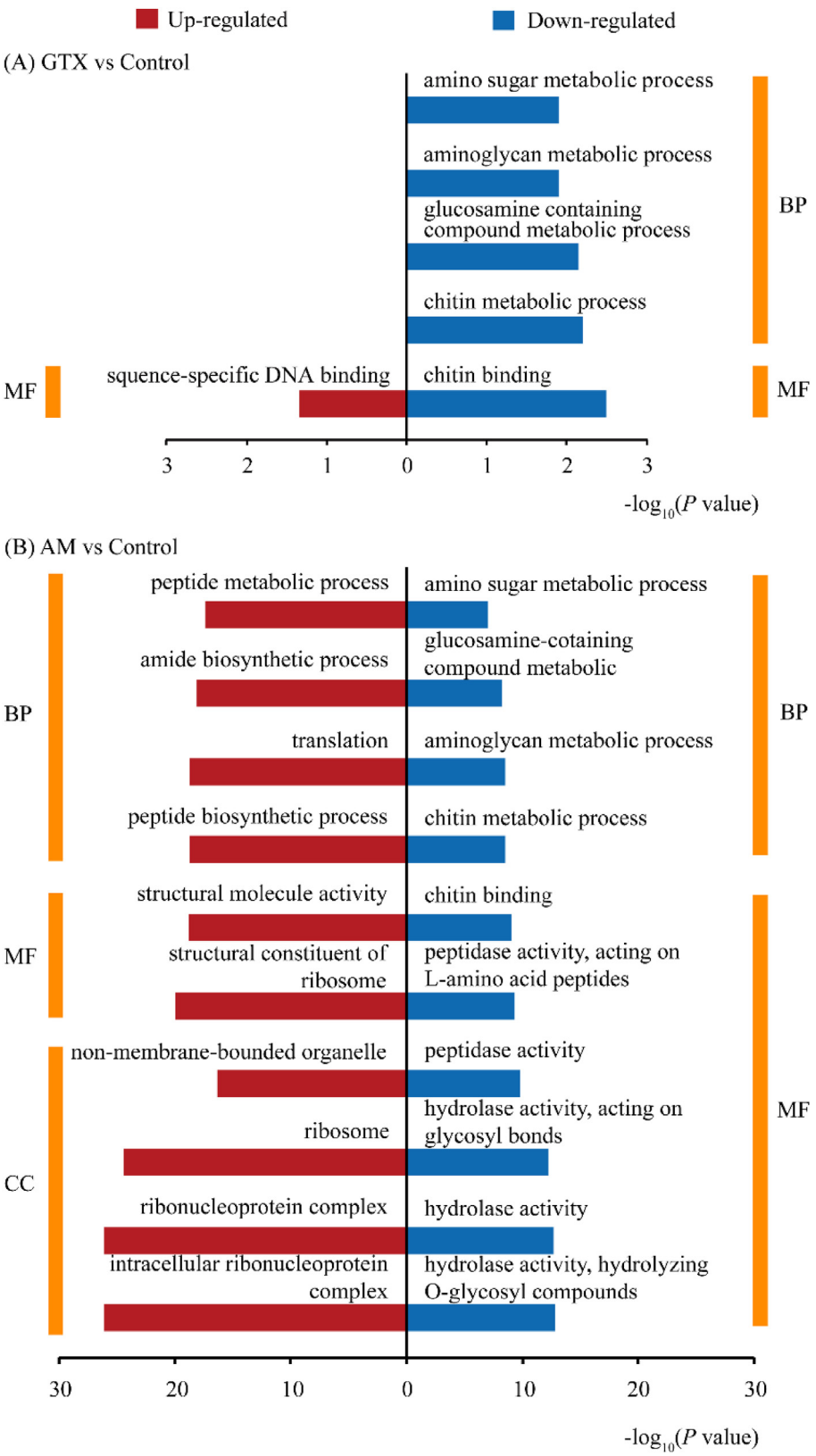

Fig. 5. Differentially expressed genes enriched in the GO pathways of *Artemia salina* after exposure to *Alexandrium minutum*. BP: biological process. MF: molecular function. CC: cellular component.

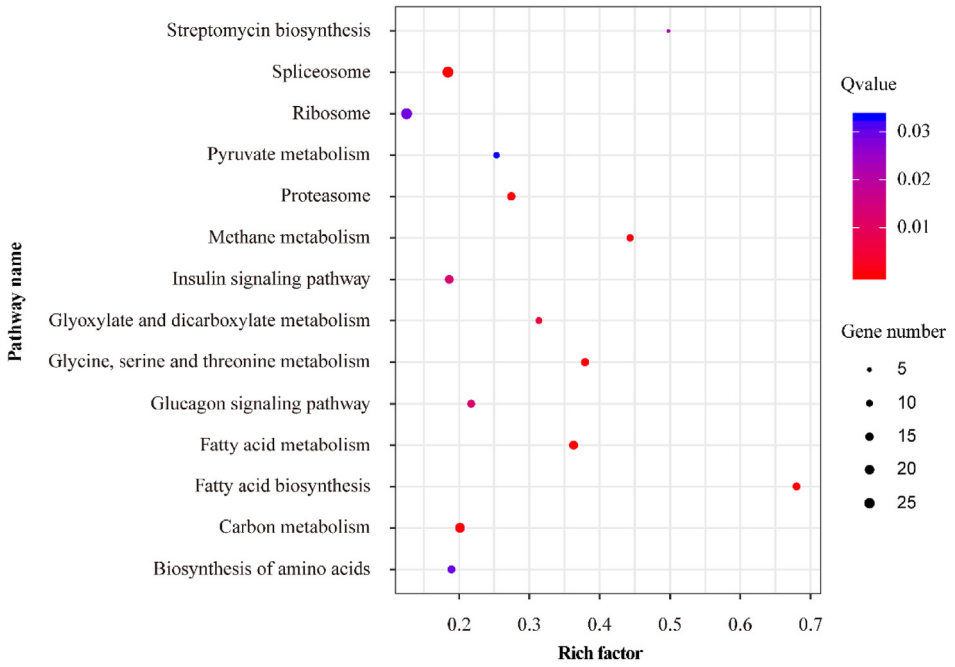

Fig. 6. Significantly enriched KEGG pathways for down-regulated DEGs of *Artemia salina* exposed to *Alexandrium minutum*.

A. salina exposed to *A. minutum* and 11, 16, 12, 9, and 25 DEGs were enriched in these pathways. In addition, 4 pathways were significantly down-regulated after exposure of *A. salina* to *A. minutum*, which indicated pancreatic metabolism, starch and sucrose metabolism, protein digestion and absorption, and ECM-receptor interaction.

3.6. RT-PCR validation

Seven genes were selected for validation of the DEG data. Compared to the control, significant up-regulation was observed for c219801_g1 after exposure of *A. salina* to GTX2/3, and for c202079_g2 and c218700_g3 after exposure to *A. minutum*. In contrast, c211368_g1 and c221389_g2 were down-regulated after exposure to GTX and c216325_g2 and c206011_g1 were down-regulated after exposure to AM. RT-PCR demonstrated similar patterns of expression as RNA-seq (Fig. 7), which independently verified the significance of these identified DEGs after exposure to *A. minutum* or GTX2/3.

4. Discussion

Recently, efforts have been made to assess effects of *A. minutum* on bivalves (Castrec et al., 2018, 2019; Borcier et al., 2017; Comeau et al., 2019), while studies of effects of *A. minutum* or its excreted extracellular products on zooplankton are limited. In the present study, trancriptomics profiling was used to demonstrate effects of exposure of *A. minutum* or GTX2/3 to *A. salina*. Responses of transcriptome in *A. salina* exposed to *A. minutum* or GTX2/3 were not exactly the same and could provide novel mechanistic insights into the potential deleterious effects of exposure to environmentally relevant doses of *A. minutum* or GTX2/3 and how *A. salina* adapts to the exposure.

4.1. Low acute toxicity on A. salina

Only slight mortality (exposure to 10^4 cells/L of *A. minutum*) or

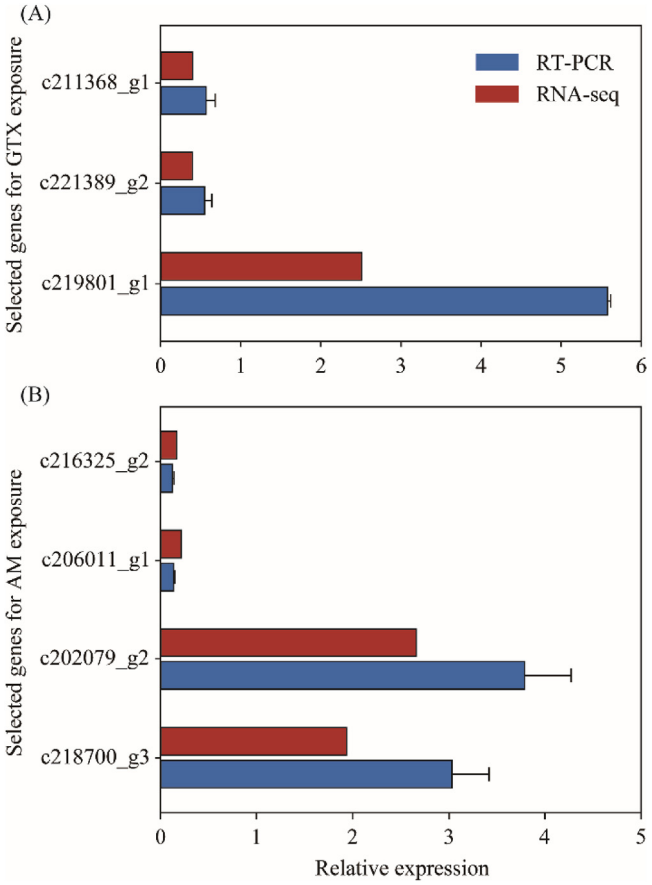

Fig. 7. RT-PCR validation of selected genes of *Artemia salina* after exposure to **(A)** GTX2/3 (GTX) or **(B)** *Alexandrium minutum* (AM). Bar indicated the average fold change of a selected unigene in exposure group relative to control. $N = 6$.

no mortality (exposure to GTX2/3 or lesser densities of *A. minutum*) were observed during the 24 h, acute toxicity test, which suggested that *A. salina* was not very sensitive to GTX2/3 or *A. minutum*. This result was in contrast to that of a previous study, in which *A. minutum* (AM-1 strain, from Taiwan) was lethal to *A. salina* at a density of 2×10^3 cells/mL (Wu et al., 2006), which indicated different toxic effects and potencies can be observed for various strains of *A. minutum*. Similar to the results presented here, some other planktonic species such as the rotifer *Brachionus plicatilis* and the shrimp *Neomysis awatschensis* were tolerant to lethality caused by the Taiwan strain of *A. minutum* for which 96 h LC_{50} were >20,000 cells/mL and 16,000 cells/mL, respectively (Chen et al., 2007). Compared to planktonic species, fishes are more sensitive to *A. minutum.* For example, the 96 h LC_{50} to larvae of *Sparus microcephalus* was 13,900 cells/mL (AMTK-2 strain, from Taiwan), and a significant effect on malformation of *S. microcephalus* eggs was observed at 7500 algal cells/mL (Jiang et al., 2010). Shellfish species, such as the oyster *Crassostrea gigas*, are the most sensitive species to *A. minutum*, and a cell density of *A. minutum* (strain AM89BM – isolated in Bay of Morlaix France) at 5000 cells/mL could reduce the energy status and motility of spermatozoa in *C. gigas* (Haberkorn et al., 2010).

4.2. Differential transcriptomic responses to A. minutum or GTX2/3

Although exposure to *A. minutum* or GTX2/3 didn't cause significant mortality to *A. salina* during the acute exposure, transcriptomic responses of *A. salina* exposed to *A. minutum* or GTX2/3 were observed. TELI developed by Gou and Gu (2011) is a useful tool to convert informative transcriptomic data into a quantitative endpoint, but relatively few studies have applied TELI-based endpoints. In the present study, despite the fact that nominal STXeq due to exposure to GTX2/3 of 90.16 ng/mL was greater than the STXeq in the *A. minutum* exposure of 72.34 ng/mL, the TELI value of *A. minutum* treated group was significantly greater, indicating that *A. minutum* caused more significant effects on transcriptomic profiles of *A. salina* than did GTX2/3 (Fig. 4B). Except for GTX toxins, *A. minutum* might excrete other toxins such as neoSTX and C-toxins (Chang et al., 1997; Hansen et al., 2003), which might have contributed to toxic potency of *A. minutum*. To date, most of the studies on PSTs isolated from *A. minutum* collected in coastal areas of China were reported to be dominated by GTXs (Bian et al., 2013; Li and Wu, 2010; Wu et al., 2005). This is consistent with the results of this study that only GTX1/4 and GTX2/3 were detected in test solutions of *A. minutum* (Table S3), thus the potential existence of other toxins could be ruled out in the present study. Another possible explanation for greater genotoxicity of *A. minutum* might be direct contact of gills of *A. salina* to *A. minutum*. Toxic potencies of various algal species, including *Prorocentrum donghaiense*, *Karenia mikimotoi* and *A. catenella* to two crustacean species, *Neomysis awatschensis* and *A. salina* have been investigated and compared by Yan et al. (2007). Those authors proposed that direct contact between gills of *A. salina,* on the outer side of the limb bases, might have lead to higher toxicity of these algal species to *A. salina* than to *K. mikinotoi* the gills of which are covered by a carapace.

Although no obvious acute lethality was observed, exposure of *A. salina* to *A. minutum* resulted in essential changes of transcriptomics (Fig. 5B), and most of the enriched GO terms were related to ribosome structure and function, including ribosome, ribonucleoprotein complex, intracellular ribonucleoprotein complex, structural constituent of ribosome, peptide biosynthetic process, translation, and peptide metabolic process. Ribonucleoproteins can perform integral functions in many biological functions, such as replication of DNA, regulating gene expression and regulating metabolism of RNA (Hogan et al., 2008). Regulation of ribosomal biogenesis is a common response to environmental stress (López-Maury et al., 2008). Genes encoding ribonucleoproteins were up-regulated in zebrafish (*Danio rerio*) after exposure to depleted uranium (Gombeau et al., 2017), which indicated that exposure could regulate cell cycle and RNA maturation in aquatic organisms. In addition, ribonucleoprotein complexes play a role of protection by regulating mRNA splicing, stability and the translational efficiency (Van Kouwenhove et al., 2011). In this study, overexpression of these genes involved in ribonucleoprotein might protect *A. salina* from stresses, such as apoptosis and reactive oxygen species (ROS) caused by exposure to *A. minutum* (Yamasaki et al., 2008).

Based on the KEGG pathway analysis (Fig. 6), up-regulated DEGs in *A. minutum* exposure group were significantly enriched in amino acids metabolism of glycine, serine and threonine and biosynthesis of amino acids and metabolism of lipids including biosynthesis and metabolism of fatty acids. Similar to regulation of ribonucleoprotein complexs, upregulation of metabolism of amino acids might also protect *A. salina* from effects of other environmental stresses. For example, under hypoxia, upregulation of amino acid transport supports synthetic reactions and maintains intracellular amounts of metabolic substrates that are essential for cell growth (Soh et al., 2007). Lipid metabolism in zooplankton species has been reported to be regulated by environmental stresses including toxic algal blooms and other chemicals. For example, in the copepod *Calanus finmarchicus*, genes related with lipid degradation were up-regulated after 2 days exposure to saxitoxin excreted by *Alexandrium fundyense* (Roncalli et al., 2016). Lipid metabolism of the water flea *Daphnia galeata* and the estuarine crab *Chiromantes dehaani* were down-regulated after exposure to an ichthyosporean gut parasite or cadmium (Cd), respectively (Liu et al., 2016; Lu et al., 2018). Biosynthesis and metabolisms of fatty acids were significantly up-regulated after exposure to *A. minutum* (Fig. 6). A total of 16 genes were enriched (Table S7). The enzymes encoded by these genes included fatty acid synthase, S-malonyltransferase, acetyl-CoA C-acetyltransferase, acyl-CoA dehydrogenase, 17 beta-estradiol 17-dehydrogenase/very-long-chain 3-oxoacyl-CoA reductase, elongation of very long chain fatty acids protein 6. These enzymes take parts in both fatty acid biosynthesis and degradation. Thus, the up-regulated gene expressions involved in lipid metabolism of *A. salina* in the present study indicated a potential requirement of energy associated with the cellular stress response.

Exposure to GTX2/3 resulted in adverse effects, mainly on chitin metabolism related pathways (Fig. 5A). Synthesis of cuticle protein as well as chitin metabolism are of great relevance for the molting process of crustacean species (Rocha et al., 2012). Inhibition of metabolism of chitin or synthesis of cuticle protein synthesis has been reported to be caused by different environmental stresses including chemical exposure to various contaminants, including benzoylphenylureas, heavy metals and bisphenol A, and this inhibition could result in disruption of molting process or disturbed sheath morphogenesis (Connon et al., 2008; Evans, 1985; Jeong et al., 2013). In addition, other environmental stresses could also regulate gene expression for proteins involved in metabolism of chitin. For example, salinity stress could up-regulate expressions of genes related to chitin metabolism in the crustaceans *A. franciscana* and *Portunus trituberculatus* (De Vos et al., 2019; Lv et al., 2013). In the present study, most of the genes enriched in GO pathways were related to metabolism of chitin and cuticle structure were down-regulated (Fig. 5A), indicating potential adverse effects of GTX on molting of *A. salina*. Disruption of molting has been documented to be related to reproduction, thus alteration of pathways associated with metabolism of chitin can ultimately affect reproduction of *A. salina* (Poynton et al., 2008). Moreover, as an external skeleton

maintaining body structure in crustacean, cuticle also functions as a defensive barrier and helps to resist environmental stressors. Thus, decreased metabolism of chitin implied weak resistibility of *A. salina* to environmental pollutants after exposure to GTX2/3 and would adversely affect fitness of *A. salina,* which could benefit blooming of phytoplankton.

4.3. Responses of defense/detoxification genes to A. minutum or GTX2/3 exposure in A. salina

Zooplankton deal with various physical factors, such as temperature and salinity, as well as chemical stressors, such as metals, hydrocarbons, and paralytic shellfish poisoning by activation of several defense/detoxification genes (Lauritano et al., 2013), including heat-shock proteins 40 and 70 (HSP40 and HSP70) (Feder and Hofmann, 1999), superoxide dismutase (SOD) (Bigot et al., 2010), glutathione *S*-transferase (GST) (Eaton and Bammler, 1999) and aldehyde dehydrogenase isoform 8 (ALDH8) (Marchitti et al., 2008). In the present study, these defense/detoxification genes have been well annotated with an E-value $\leq 1e^{-5}$ and an identity value > 60% in *A. salina* (**Supplemental material,** Table S8). Compared to the control group, expression of an unigene encoding for HSP70 was significantly up-regulated by exposure to *A. minutum* or GTX2/3 for 24 h (Table S9). However, expressions of unigenes for HSP40, SOD, GST, and ALDH8 were not significantly affected by exposure to *A. minutum* or GTX2/3, relative to the control. This might be attributed to the relatively, short-term, acute exposure in this study. Similarly, after 3 days of feeding on *Karenia brevis*, gene expressions of HSP40, HSP70, SOD, GST, and ALDH isoforms in copepod *Calanus helgolandicus* were not significantly changed. However, after longer exposure of 5 or 8 days, HSP40, GST, and ALDH isoforms 2, 3, 6, 7, and 9 were significantly down-regulated while HSP70 was significantly up-regulated (Lauritano et al., 2013). Although not statistically significant, in this study, lesser expressions of SOD was also observed after 24 h of exposure to *A. minutum*. HSP70 is involved in initiation of cellular process in response to stress and help to keep proteins properly folded (reviewed by Maiti, 2015). Exposure to *A. minutum* and its excreted saxitoxins could up-regulate expression of HSP70 in marine bivalves, such as in *Chlamys farreri* and *Crassostrea gigas* (Mello et al., 2013; Hu et al., 2019). In the present study, up-regulated expression of the HSP70 gene was an adaptive response of *A. salina* to effects of exposure to *A. minutum* or GTX2/3. This result also suggested that HSP70 has potential to be used as a non-specific, first tier biomarker of exposure to *A. minutum* or GTX2/3.

4.4. Environmental relevance

Numbers of *A. minutum* in coastal environment can be greater than the populations of algal cells employed in the present study, especially during bloom conditions (Anderson et al., 2012). Therefore, the effects of exposure to 10^4 cells/mL *A. minutum* might, to some extent, result in realistic responses of *A. salina* to *A. minutum* exposure in real environments. Although *A. minutum* did not cause significant lethality of *A. salina* during a 24 h acute exposure, it did cause essential changes of transcriptomics in *A. salina*, which suggests adaptive responses that might have adverse effects on overall fitness due to exposure to environmentally realistic levels of *A. minutum*. In spite of the wide application of *A. salina* in ecotoxicological studies, information on the transcriptome of this species was still limited, with only a recent study that investigated salt tolerance of *A. franciscana* and reported its annotated transcriptome (De Vos et al., 2019). Here, our present study, for the first time, reported the transcriptome of *A. salina* and identified altered pathways in response to exposure of toxic algae (*A. minutum*) or the excreted toxins of algae, such as GTX2/3 at environmentaly relevant concentrations.

Exposures to varying compositions and proportions of toxins excreted by various strains of *A. minutum* or during or at different development stages could result in different responses. Therefore, both physiological and transcriptomic responses of *A. salina* when exposed to *A. minutum* could be different if *A. minutum* of another strain or at another developmental stage was applied in the study. In spite of this limitation, the results of the present study could still provide the opportunity to identify potential biomarkers during algal blooms in coastal environment.

5. Conclusions

Results of the study presented here demonstrated that based on lethality, neither larvae nor adult *A. salina* were very sensitive to exposure to *A. minutum* or GTX2/3 for 24 h. Overall, transcriptional results of the study represented the first report of the application of next generation sequencing techniques to investigate the transcriptomic response of *A. salina* exposed to *A. minutum* or GTX2/3 at environmental realistic densities or concentrations. Exposure to either *A. minutum* or GTX2/3 for 24 h resulted in significant changes in transcriptomic profiles of *A. salina.* Based on TELI values *A. minutum* caused greater effects at the transcriptional level than did GTX2/3. Transcriptomic responses of *A. salina* exposed to *A. minutum* or GTX2/3 were quite different, and enriched GO terms and KEGG pathways in *A. salina* revealed different effects of *A. minutum* or GTX2/3. Exposure to *A. minutum* regulated gene expression of ribonucleoprotein complex and metabolism of amino acid and lipid in *A. salina*. Over-expression of these genes might act as a protective role in *A. salina* from *A. minutum* induced stresses, while GTX2/3 exposure could inhibit the expression of chitin metabolism related genes which might result in disruption of molting process or disturbed sheath morphogenesis.

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Appendix A. Supplementary data

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Supplementary material for

Transcriptomic responses of *Artemia salina* exposed to an environmentally relevant dose of *Alexandrium minutum* cells or Gonyautoxin2/3

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1. Detailed analysis parameters for LC-MS/MS

Table S1. The detailed analysis parameters for LC-MS/MS

Target compound	Precursor ion (m/z)	Product ion (m/z)	DP (eV)	EP (eV)	CE (eV)	CXP (eV)
GTX2	396.1	316.1*	66	9	18	16
		298.1	66	9	28	16
		378.1	66	9	17	17
GTX3	396.1	316.1	66	9	18	16
		298.1*	66	9	28	16
		378.1	66	9	17	17
GTX1	412.1	332.1*	66	8	14	9
		313.9	66	8	18	10
		394.0	66	8	28	9
GTX4	412.1	332.1	66	8	14	9
		313.9*	66	8	18	10
		394.0	66	8	28	9
GTX5	380.1	300.1*	66	8	23	20
		282.1	66	8	19	18
		257.1	66	8	34	20
NeoSTX	316.1	298.0*	66	8	32	16
		220.0	66	8	42	13
		138.1	66	8	27	22
STX	300.1	204.0*	66	8	33	16
		179.0	66	8	34	16
		138.2	66	8	39	15
dcSTX	257.1	138.1*	66	10	30	12
		126.0	66	9	35	14
		180.0	66	9	25	15
dcGTX2	353.3	254.9*	66	8	16	20
		272.9	66	8	18	20
		335.1	66	8	31	20
dcGTX3	353.3	254.9	66	8	16	20
		272.9*	66	8	18	20
		335.1	66	8	31	20
C1	396.2	316.2*	90	10	22	11
		298.2	90	10	28	11
C2	396.2	316.2	90	10	22	11
		298.2*	90	10	28	11
dcNeoSTX	273.1	180.0*	66	8	30	14
		126.0	66	8	32	11
		162.2	66	8	37	16

* product ion for quantification

2. Determination of transcriptional effect level index (TELI)

TELI converts the information-rich toxicogenomic data into an integrated endpoint index, that can represent overall alteration of transcriptions alteration (Gou and Gu 2011). The TELI considers and incorporates three factors: (1) the number and identify of genes that exhibited altered expression, (2) the magnitude of altered gene expression for each gene response to the exposure, and (3) the time factor. Here, a single duration of exposure was used for all treatments so it can be set to a constant unity value of 1.0. Thus, the TELI value is calculated using the following equation:

$$\mathrm{TELI}_{(\mathrm{genei})} = e^{|\ln(\mathrm{I})|} - e^{|\ln(1)|} \quad \text{(Equation 1)}$$

$$\mathrm{TELI}_{(\mathrm{total})} = \sum_{\mathrm{gene\ (i=1)}}^{\mathrm{genei\ (i=n)}} (\mathrm{TELI}_{\mathrm{genei}}) \quad \text{(Equation 2)}$$

Where, I is the gene expression change; i is the number of genes in the transcriptomics data, and control gene expression level (I = 1) is subtracted from each data point.

3. Primer sequences for RT-PCR

Table S2. Primer sequences used in RT-PCR analysis[1]

Unigene name	NR database annotation	E-value	Primer sequences (5′ →3′)
c219801_g1	Heat shock protein 70	0	F: GACTAATTGGCCGTCGATTTGATGAC
			R: ATGATTCAAGAGAATTCTTGGCAGCG
c221389_g2	Zinc metalloproteinase	$2.62e^{-14}$	F: GTGCTTCATGAGCTGTACCATGCTCT
			R: GCTTGTAGTTGATTCTTCGAACCTGT
c211368_g1	Hypothetical protein DAPPUDRAFT_240263	$6.69e^{-155}$	F: ATGGACACCGAAGTAGTACTTGTCAC
			R: GTCATCTCCATGGGATACTCCTCTAG
c218700_g3	L-lactate dehydrogenase isoform X2	$4.20e^{-80}$	F: TAGTGAGCTGGTCCTCGTTGAT
			R: GTTGTGGAGATCTGCTAGCTCAGC
c202079_g2	Guanine nucleotide binding protein, q polypeptide	0	F: TGCCTGACTACTTATAGAATAGGCTG
			R: GCGTATATTCTCAGTATCTGTTGCAC
c206011_g1	Myosin light chain	$2.74e^{-111}$	F: GTCGGTTACCATGGTCTAAACTTAGA
			R: TGGAGCTTCGTTGATCATCTCATC
c216325_g2	Myosin heavy chain, muscle isoform X13	0	F: CCAAGACAGTTCGTAACGACAACT
			R: GGCTTTGAGTTTGTTGAGCTGATC

1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as housekeeping gene. The primer sequences (5’ → 3’) were F: GTTGATGGCAAACTCGTCATA; R: CCACCTTCCAAGTGAGCATTA, according to Chen and Ge (2009).

4. Calculation of saxitoxin equivalents (STXeq)

The European Food Safety Authority (EFSA) (2009), defines toxicity equivalency factors (TEFs) of STX-group toxins of 1.0 for GTX1, 0.4 for GTX2, 0.6 for GTX3, and 0.7 for GTX4. Thus, the STXeq was calculated using the following equation in the present study:

$$STXeq = 1.0 \times Conc._{(GTX1)} + 0.4 \times Conc._{(GTX2)} + 0.6 \times Conc._{(GTX3)} + 0.7 \times Conc._{(GTX4)}$$

where Conc. is the measured concentration of a specific gonyautoxin congener in exposure solutions from *Alexandrium minutum* group.

Table. S3 Gonyautoxin concentrations measured in exposure solutions from *Alexandrium minutum* group by LC-MS/MS (N = 3)

Toxin	GTX1	GTX2	GTX3	GTX4	STXeq[1]
Concentrations (ng/mL)	11.88 ± 1.6	83.5 ± 3.5	42.23 ± 2.4	2.46 ± 0.7	72.34 ± 3.2

1. STXeq was calculated based on the measured concentrations in exposure solutions from *Alexandrium minutum* group

5. Summary statistics of transcriptome sequencing

Table S4. Summary statistics of transcriptome sequencing for *Artemia salina* from control group (C1-3), gonyautoxin2/3 exposure group (GTX 1-3) and *Alexandrium minutum* exposure group (AM 1-2)

Sample name	Raw reads	Clean reads	Clean bases	Q20(%)	Q30(%)	GC content(%)	Transcript number	Unigene number	Mean length of unigenes (bp)
C1	43897488	43579224	6.45G	97.61	93.88	40.87	599,286	515,196	444.58
C2	42998414	42679336	6.31G	97.61	94.10	40.87			
C3	45808962	45521926	6.72G	97.89	95.13	41.06			
GTX1	40184250	39917110	5.91G	97.85	94.65	40.70			
GTX2	41180378	40846128	6.04G	97.57	94.06	41.00			
GTX3	43576218	43228920	6.38G	97.74	94.36	41.23			
AM1	41520364	41131410	6.08G	97.89	93.92	41.88			
AM2	42830456	42366304	6.19G	97.90	94.90	42.57			

6. Annotation of unigenes

Table S5. Annotation of unigenes in different databases

Databases	Number of unigenes	Percentage (%)
NR	69,676	13.52
GO	142045	27.57
KEGG	4860	0.94
eggNOG	66,007	12.81
Swiss-Prot	60,933	11.83
In all database	2895	0.56

7. GO enrichment analysis of differentially expressed genes

Table S6. GO enrichment analysis

Group	Category	GO Term	Corrected *P* value	Num. of Genes
GTX vs Control, upregulated	Molecular function	GO:0043565 sequence-specific DNA binding	0.04567	2
GTX vs Control,	Molecular function	GO:0008061 chitin binding	0.00316	2
downregulated	Biological process	GO:0006030 chitin metabolic process	0.00619	2
	Biological process	GO:1901071 glucosamine-containing compound metabolic process	0.00708	2
	Biological process	GO:0006022 aminoglycan metabolic process	0.01243	2
	Biological process	GO:0006040 amino sugar metabolic process	0.01243	2
AM vs Control, upregulated	Cellular component	GO:0030529 intracellular ribonucleoprotein complex	7.59E-27	492
	Cellular component	GO:1990904 ribonucleoprotein complex	7.59E-27	492
	Cellular component	GO:0005840 ribosome	3.55E-25	374
	Cellular component	GO:0043228 non-membrane-bounded organelle	4.31E-17	564
	Cellular component	GO:0043232 intracellular non-membrane-bounded organelle	4.31E-17	564
	Cellular component	GO:0044391 ribosomal subunit	3.16E-16	218
	Cellular component	GO:0044445 cytosolic part	5.85E-13	213
	Cellular component	GO:0022626 cytosolic ribosome	2.34E-12	183

Cellular component	GO:0032991 macromolecular complex	7.6E-12	780
Cellular component	GO:0015934 large ribosomal subunit	2.5E-08	125
Cellular component	GO:0022625 cytosolic large ribosomal subunit	7.99E-08	112
Cellular component	GO:0005622 intracellular	2.46E-07	1449
Cellular component	GO:0005829 cytosol	5.78E-07	314
Cellular component	GO:0000502 proteasome complex	5.94E-07	64
Cellular component	GO:0005737 cytoplasm	1.81E-06	967
Cellular component	GO:0044464 cell part	5.41E-06	1524
Cellular component	GO:0005623 cell	9.96E-06	1531
Cellular component	GO:0044424 intracellular part	0.0000327	1350
Cellular component	GO:0044444 cytoplasmic part	0.00013	761
Cellular component	GO:0015935 small ribosomal subunit	0.00036	91
Cellular component	GO:0022627 cytosolic small ribosomal subunit	0.00303	70
Cellular component	GO:0043226 organelle	0.01976	1060
Cellular component	GO:0043229 intracellular organelle	0.02873	1014
Molecular function	GO:0003735 structural constituent of ribosome	1.19E-20	297
Molecular function	GO:0005198 structural molecule activity	1.67E-19	332

Molecular function	GO:0005200 structural constituent of cytoskeleton	0.00165	28
Molecular function	GO:0032561 guanyl ribonucleotide binding	0.00199	118
Molecular function	GO:0019001 guanyl nucleotide binding	0.00215	120
Molecular function	GO:0005525 GTP binding	0.00271	116
Molecular function	GO:0036402 proteasome-activating ATPase activity	0.01028	12
Molecular function	GO:0017025 TBP-class protein binding	0.04703	12
Biological process	GO:0043043 peptide biosynthetic process	1.77E-19	405
Biological process	GO:0006412 translation	1.81E-19	404
Biological process	GO:0043604 amide biosynthetic process	7.2E-19	411
Biological process	GO:0006518 peptide metabolic process	4.46E-18	410
Biological process	GO:0043603 cellular amide metabolic process	2.46E-16	419
Biological process	GO:1901566 organonitrogen compound biosynthetic process	7.61E-12	481
Biological process	GO:0010467 gene expression	5.19E-10	540
Biological process	GO:0044271 cellular nitrogen compound biosynthetic process	1.03E-09	499
Biological process	GO:0044267 cellular protein metabolic process	2.2E-09	631
Biological process	GO:1901564 organonitrogen compound metabolic process	9.95E-09	596
Biological process	GO:0034645 cellular macromolecule biosynthetic process	9.95E-07	450

	Biological process	GO:0009059 macromolecule biosynthetic process	1.67E-06	453
	Biological process	GO:0019538 protein metabolic process	8.88E-06	676
	Biological process	GO:0034641 cellular nitrogen compound metabolic process	0.00163	786
	Biological process	GO:0022613 ribonucleoprotein complex biogenesis	0.0086	163
	Biological process	GO:0006807 nitrogen compound metabolic process	0.00964	844
	Biological process	GO:0044260 cellular macromolecule metabolic process	0.01885	850
	Biological process	GO:0042273 ribosomal large subunit biogenesis	0.02497	60
	Biological process	GO:0006457 protein folding	0.03337	89
	Biological process	GO:0042254 ribosome biogenesis	0.03428	139
	Biological process	GO:0060261 positive regulation of transcription initiation from RNA polymerase II promoter	0.04113	12
	Biological process	GO:0045899 positive regulation of RNA polymerase II transcriptional preinitiation complex assembly	0.04113	12
	Biological process	GO:2000144 positive regulation of DNA-templated transcription, initiation	0.04113	12
AM vs Control, downregulated	Cellular component	GO:0016459 myosin complex	0.000000217	7

Cellular component	GO:0015629 actin cytoskeleton	0.00061	8
Cellular component	GO:0005887 integral component of plasma membrane	0.01356	8
Cellular component	GO:0031226 intrinsic component of plasma membrane	0.01543	8
Molecular function	GO:0004553 hydrolase activity, hydrolyzing O-glycosyl compounds	1.47E-13	17
Molecular function	GO:0016787 hydrolase activity	1.96E-13	76
Molecular function	GO:0016798 hydrolase activity, acting on glycosyl bonds	6.39E-13	20
Molecular function	GO:0008233 peptidase activity	1.75E-10	31
Molecular function	GO:0070011 peptidase activity, acting on L-amino acid peptides	4.58E-10	26
Molecular function	GO:0008061 chitin binding	9.1E-10	9
Molecular function	GO:0004181 metallocarboxypeptidase activity	0.000000972	5
Molecular function	GO:0008236 serine-type peptidase activity	0.00000224	12
Molecular function	GO:0017171 serine hydrolase activity	0.00000224	12
Molecular function	GO:0004252 serine-type endopeptidase activity	0.00000326	9
Molecular function	GO:0008237 metallopeptidase activity	0.00000983	10
Molecular function	GO:0004568 chitinase activity	0.0000489	5
Molecular function	GO:0004180 carboxypeptidase activity	0.00013	6

Molecular function	GO:0008238 exopeptidase activity	0.00023	8
Molecular function	GO:0043169 cation binding	0.0005	33
Molecular function	GO:0046872 metal ion binding	0.00063	32
Molecular function	GO:0009881 photoreceptor activity	0.00076	4
Molecular function	GO:0008235 metalloexopeptidase activity	0.00079	5
Molecular function	GO:0004175 endopeptidase activity	0.00111	12
Molecular function	GO:0003779 actin binding	0.00569	7
Molecular function	GO:0008422 beta-glucosidase activity	0.00815	3
Molecular function	GO:0008270 zinc ion binding	0.00827	11
Molecular function	GO:0046914 transition metal ion binding	0.0113	14
Molecular function	GO:0004930 G-protein coupled receptor activity	0.01221	5
Molecular function	GO:0099600 transmembrane receptor activity	0.01768	7
Molecular function	GO:0003824 catalytic activity	0.01772	110
Molecular function	GO:0004888 transmembrane signaling receptor activity	0.03819	6
Biological process	GO:0006030 chitin metabolic process	3.21E-09	9
Biological process	GO:0006022 aminoglycan metabolic process	3.4E-09	10
Biological process	GO:1901071 glucosamine-containing compound metabolic process	6.35E-09	9

	Biological process	GO:0006040 amino sugar metabolic process	0.0000001	9
	Biological process	GO:0006026 aminoglycan catabolic process	0.00000537	6
	Biological process	GO:1901136 carbohydrate derivative catabolic process	0.0000326	7
	Biological process	GO:0006032 chitin catabolic process	0.0000824	5
	Biological process	GO:0046348 amino sugar catabolic process	0.00011	5
	Biological process	GO:1901072 glucosamine-containing compound catabolic process	0.00011	5
	Biological process	GO:0007602 phototransduction	0.00128	4
	Biological process	GO:0009583 detection of light stimulus	0.00344	4
	Biological process	GO:0009582 detection of abiotic stimulus	0.01171	4
	Biological process	GO:0009581 detection of external stimulus	0.01171	4
	Biological process	GO:0018298 protein-chromophore linkage	0.0293	4

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8. KEGG enrichment analysis of differentially expressed genes

Table S7. KEGG enrichment analysis

Group	Pathway ID	Pathway	DEG number	Total number	*P* value	FDR
GTX vs Control, downregulated	ko04213	Longevity regulating pathway - multiple species	1	32	0.0127654	0.0255308
	ko04141	Protein processing in endoplasmic reticulum	1	109	0.0431461	0.0431461
AM vs Control, upregulated	ko00061	Fatty acid biosynthesis	11	16	1.17637E-09	2.03512E-07
	ko01212	Fatty acid metabolism	16	43	3.09548E-08	2.67759E-06
	ko00260	Glycine, serine and threonine metabolism	12	31	1.04898E-06	6.04912E-05
	ko00680	Methane metabolism	9	20	5.59338E-06	2.23263E-04
	ko03040	Spliceosome	25	128	6.72346E-06	2.23263E-04
	ko03050	Proteasome	13	46	2.07354E-05	0.0005979
	ko01200	Carbon metabolism	19	90	2.81543E-05	0.0006958
	ko00630	Glyoxylate and dicarboxylate metabolism	8	25	0.00032932	0.0063302
	ko04910	Insulin signaling pathway	14	72	0.00077384	0.0127188
	ko04922	Glucagon signaling pathway	11	49	0.00080871	0.0127188
	ko00521	Streptomycin biosynthesis	4	8	0.00172947	0.0230153
	ko03010	Ribosome	25	183	0.00233229	0.0288204

	ko01230	Biosynthesis of amino acids	11	56	0.00256498	0.0295828
	ko00620	Pyruvate metabolism	7	27	0.00305812	0.0330659
AM vs Control, downregulated	ko04972	Pancreatic secretion	6	40	5.95714E-05	0.0034863
	ko00500	Starch and sucrose metabolism	5	26	7.41762E-05	0.0034863
	ko04974	Protein digestion and absorption	4	21	0.00042720	0.0133856
	ko04512	ECM-receptor interaction	3	14	0.00166851	0.0392101

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9. Expressions of defense/detoxification genes in adult *A. salina* after exposure

Table S8. Annotated defense/detoxification genes in *A. salina*

Gene symbol	Unigene ID	Length	NR database annotation	E value	Identity
HSP40	c211689_g2	1517	gi|821371453|gb|AKH40961.1| type I heat shock protein 40 [*Artemia franciscana*]	0	97.77%
HSP70	c219801_g1	1811	gi|683687200|gb|AIN41694.1| heat shock protein 70 [*Artemia sinica*]	0	91.21%
SOD	c198031_g1	1119	gi|1022772851|gb|KZS17008.1| Superoxide dismutase [*Daphnia magna*]	2.88E-110	77.39%
GST	c200781_g2	1251	gi|478859730|gb|AGJ70295.1| glutathione S-transferases [*Macrobrachium nipponense*]	8.86E-68	67.97%
ALDH8	c217769_g1	5373	gi|1022777183|gb|KZS20768.1| Aldehyde dehydrogenase family 8 member A1 [*Daphnia magna*]	0	62.37%

Table S9. Expression levels of defense/detoxification genes in adult *A. salina* after exposure to *A. minutum* or GTX2/3 for 24 hours

Gene symbol	Unigene ID	*A. minutum* exposure		GTX2/3 exposure	
		Fold change	*P* value	Fold change	*P* value
HSP40	c211689_g2	1.332	0.279	1.211	0.306
HSP70	c219801_g1	**2.338** ↑	0.002	**2.510** ↑	1.12E-06
SOD	c198031_g1	**0.657** ↓	0.154	0.747	0.139
GST	c200781_g2	0.866	0.712	0.927	0.817
ALDH8	c217769_g1	0.959	0.844	1.089	0.694

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