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Environmental Stress Responses and Experimental Handling Artifacts of a Model Organism, the Copepod *Acartia tonsa* (Dana)

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Handling animals during experiments potentially affects the differential expression of genes chosen as biomarkers of sub-lethal stress. RNA sequencing was used to examine whole-transcriptome responses caused by laboratory handling of the calanoid copepod, *Acartia tonsa*. Salinity shock ($S = 35$ to $S = 5$) was used as positive stress control; individuals not exposed to handling or other stressors served as negative stress control. All copepods were grown from eggs to adults without being handled or exposed to any stressors prior the experiment. Survival of nauplii and adults was estimated for up to 10 min of exposure to handling stress and salinity shock. Only adults exhibited decreased survival (44 ± 7% with 10 min of exposure) in response to handling stress and were selected for definitive experiments for RNA sequencing. After 10 min of experimental exposures to handling stress or salinity shock, adults were incubated for 15 min or 24 h at normal culture conditions. A small number of significantly differentially expressed genes (DEGs) were observed 15 min after exposure to handling stress (2 DEGs) or salinity shock (7 DEGs). However, 24 h after exposure, handling stress resulted in 276 DEGs and salinity shock resulted in 573 DEGs, of which 174 DEGs were overlapping between the treatments. Among the DEGs observed 24 h after exposure to handling stress or salinity shock, some commonly-used stress biomarkers appeared at low levels. This suggests that a stress-response was induced at the transcriptional level for these genes between 15 min and 24 h following exposure. Since handling stress clearly affects transcriptional patterns, it is important to consider handling when designing experiments, by either including additional controls or avoiding focus on impacted genes. Not considering handling in gene expression studies can lead to inaccurate conclusions. The present study provides a baseline for studying handling stress in future studies using this model organism and others.

Keywords: stress, handling, salinity, *Acartia tonsa,* copepod, RNA sequencing, environmental monitoring

INTRODUCTION

Copepods provide a principal link in the transfer of energy from phytoplankton to higher trophic levels in the marine food webs, and are preferred prey for predators, such as juvenile fishes and shrimps (Turner, 2004). Given the high natural abundance of copepods and their importance for marine ecosystems, understanding how stressors affect copepods is a concern for estuarine and marine ecology. Copepods are widely used in environmental monitoring as indicators of ecosystem health (Beaugrand, 2009). Hence, stress responses of copepods used for diagnostic (e.g., ecotoxicology testing) or experimental purposes might result in wrong conclusions when interpreting or extrapolating results from procedures that entail experimental handling.

Transcriptional biomarkers that are commonly used to indicate sub-lethal effects of stress include detoxification enzymes (i.e., *cytochrome P450* and *Glutathionine-S-transferase*), as well as stress-related proteins, or chaperones, that protect macromolecules from damage (Davies and Vethaak, 2012; Amiard-Triquet and Berthet, 2015). In general, it is an overlooked issue that some of these biomarkers may respond to stress associated with handling, capture, collection, and other events in the experimental setup of both laboratory and field studies. Failure to consider the effects of experimental handling on gene expression during studies of environmental stress could cause erroneous conclusions about the data, by either increasing the risk of false positive results or by masking treatment-specific effects. Experimentally-induced and handling-related stress has been extensively studied in larger crustaceans (Fotedar and Evans, 2011). To our knowledge, only a few studies, e.g., Aruda et al. (2011) and Rahlff et al. (2017), have examined handling stress in copepods with targeted methods, which entail the evaluation of specific transcriptional biomarkers selected to evaluate certain stressors, typically by real-time quantitative PCR.

The aim of this study is to examine the transcriptome-wide effects of handling stress on the calanoid copepod, *Acartia tonsa*. Because of the growing interest in *A. tonsa* as a model species for experimental studies, as well as an indicator species for environmental monitoring, and a valuable live-feed species for aquaculture industries, there are important issues for selecting appropriate biomarkers and establishing an accurate baseline description of a non-stressed copepod (Kwok et al., 2015). In both laboratory and field studies, plankton nets are commonly used for collection and size separation of copepod life stages (Uye and Kuwata, 1983; Rahlff et al., 2017). Because of this, the use of plankton nets was used to represent handling stress in this study.

A. tonsa is a robust species that, when acclimated, can persist in salinities ranging from 1 to 72 S, with an optimal salinity around 15 to 22 S (Holste and Peck, 2006). Even though *A. tonsa* is more tolerant to salinity variation than other *Acartia* species, abrupt change in salinity has been documented as a significant stressor (Lance, 1964; Chinnery and Williams, 2004; Calliari et al., 2006). Abrupt changes in salinity that exceed 10-15 S relative to the ambient level of *A. tonsa* have been shown to decrease survival more than 50% (Cervetto et al., 1999). For a positive-stress control, we used an extreme salinity shock from $S = 35$ to $S = 5$ to provoke a response at both the transcriptional and physiological levels.

In addition to the overall lack of data in relation to handling stress, there is no information regarding the extent to which handling will cause changes at the transcriptional level. Since this is a first approach to examine transcriptome-wide handling stress, the treatments used may be considered somewhat "extreme," and are designed to ensure a response at the transcriptional level. The intention of the present study is to establish a foundation for future studies for this model species and others, in which handling stress can be described in greater detail.

MATERIALS AND METHODS

Stock Culture

The strain of *A. tonsa* (identity code: DFH.AT1*)* has been in culture since 1981, when it was collected from Øresund (56° N; 12° E; Denmark) (Støttrup et al., 1986). The strain has been cultivated at Roskilde University (Denmark) in 60 L polyethylene tanks for >20 years under stable conditions (0.2 μm filtered seawater, $S = 35$, 17°C, oxygen > 60%, dim lighting). The diet consisted of the microalgae, *Rhodomonas salina* (identity code: K-1487). *R. salina* is cultivated in 2 L round-bottom flasks under stable temperature (17°C), with constant aeration and light (PAR ∼ 80 μE m^{-2} s^{-1}). The algal culture is diluted daily with Guillard's F/2 enrichment solution (Guillard and Ryther, 1962).

Experimental Cultures

The experimental design comprised three treatments: control (no stress) (**Figure 1A**), handling stress (**Figure 1B**) and salinity shock (**Figure 1C**). Embryos of mixed age and stage were harvested from three stock cultures and transferred to 2 L Nalgene© polycarbonate bottles (Thermo Fisher Scientific, USA) containing 0.2 μm filtered seawater ($S = 35$), where they were incubated at 17°C for hatching. For each of the treatments triplicate cultivation flasks were set up as biological replicates. During cultivation, as well as during all experimental treatments, the flasks were kept at 17 ± 1°C, with dim light and gentle aeration. *R. salina* were fed to the copepods in excess (>800 μg C L^{-1}; Berggreen et al., 1988) daily. Oxygen content was measured daily with a hand-held oxygen-probe (Handy Polaris 2, OxyGuard International A/S, Denmark) and exhibited values ranged from 6.9 to 7.5 mg O_2 L^{-1}.

Nauplii (body length: 130 ± 16 μm, 4 days of development $n = 45$) and adults (prosome length: 730 ± 54 μm, 15 days of development) were monitored for survival. Adults (764 ± 42 μm, 15 days of development $n = 48$) used for RNA sequencing were grown from eggs to the desired life stage without being handled. Prosome lengths were measured by photographing the copepods with a Nikon SM218 microscope with 13.5x magnification, mounted with a Nikon Digital sight DS-U3 camera (20×magnification) and subsequently analyzing the images using the software package NIS-Elements BR 4.40 (Nikon Instruments Europe, B.V., The Netherlands).

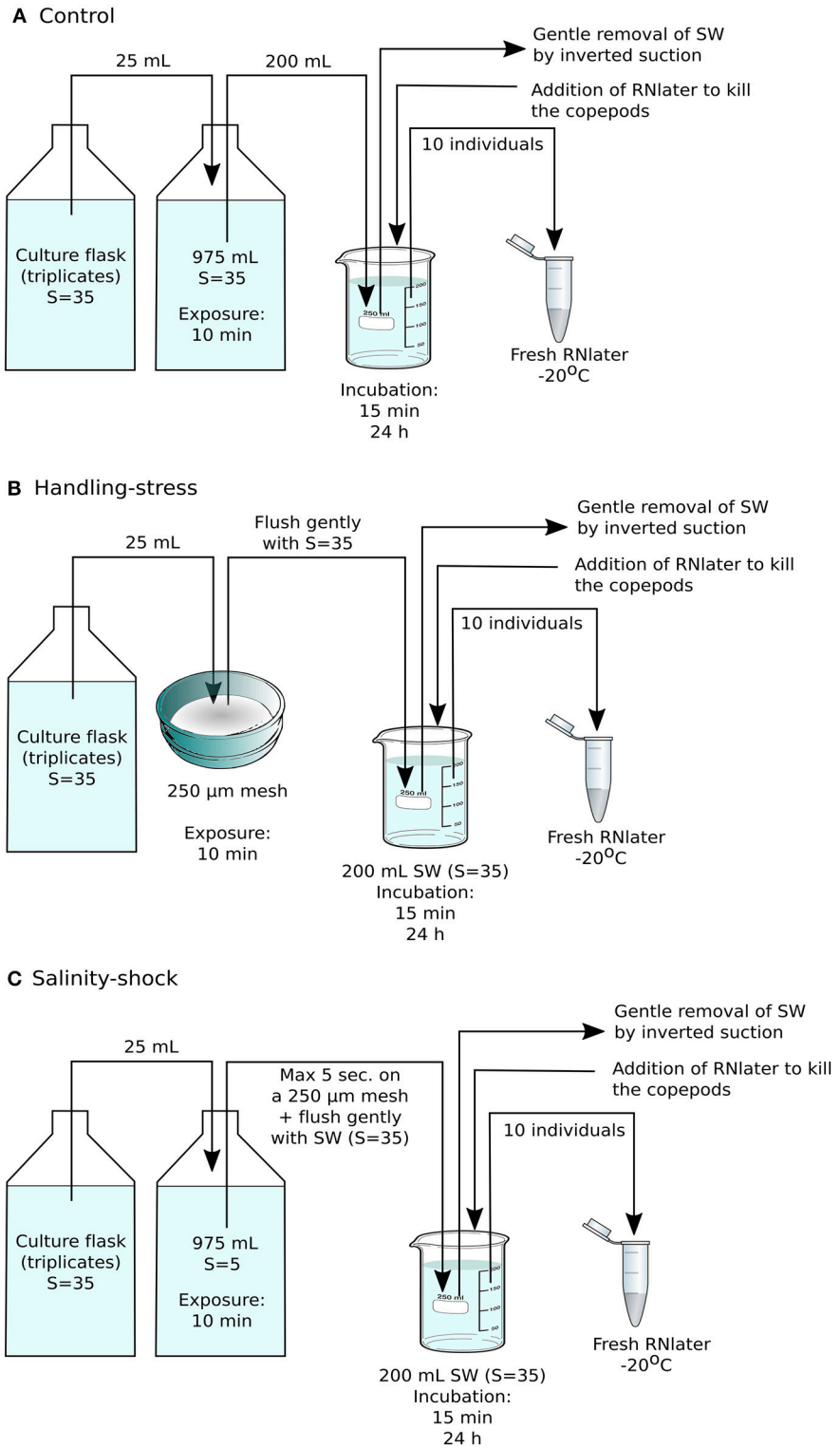

FIGURE 1 | Experimental setup for RNA sequencing. **(A)** The control consisted of *Acartia tonsa* grown from eggs to adults prior to the experiment without being handled, followed by incubation at regular culture conditions for 15 min or 24 h, prior to fixation with RNAlater. **(B)** In the handling stress treatment, adult individuals were placed on Nitex plankton net material with mesh size of 250 µm, and then kept at stock culture conditions for 15 min or 24 h before fixation with RNA later. **(C)** The salinity shock treatment consisted of animals exposed to salinity $S = 5$ for 10 min, followed by incubation at stock culture conditions for 15 min or 24 h. Each treatment was performed in triplicate, with each sample containing 10 adult individuals of *A. tonsa*.

Experimental Design

The experimental design was repeated twice, once to estimate survival and a second time for RNA sequencing (**Figure 1**). The initial determination of survival was used to identify both the life-stage and the exposure time for the definitive experiment for RNA sequencing.

For the control, nauplii or adults were removed from the triplicate cultures in Nalgene© bottles using a 25 mL automatic pipettor (NS 29.2/32,Witeg, Germany) and transferred to 1 L glass beakers containing 975 mL seawater, with the same conditions as described for the stock culture. The copepods were held in the control treatment for 25 s or 10 min before being transferred to new glass beakers. The copepods exposed to salinity shock were transferred to seawater with a salinity of $S = 5$; copepods exposed to handling stress were transferred to a Nitex plankton net (mesh size 54 μm for nauplii, 250 μm for adults) that was not submerged in water. For salinity shock and handling stress, the copepods were exposed for 25 sec, 1, 5, or 10 min before being transferred to glass beakers containing seawater with the same conditions as the stock culture. To distinguish between alive and dead, neutral red stain was added (15 mg/L seawater; Elliott and Tang, 2009). After rinsing with distilled water, dead and alive copepods were counted and survival was estimated. All three treatments were performed with 4 replicates.

Based on the results of the initial survival experiment, the definitive experiment for RNA sequencing was designed to include adult copepods and an exposure time of 10 min for handling stress or salinity shock, after which copepods were incubated for 15 min or 24 h at stock culture conditions. Seawater was then gently removed by inverted suction and the copepods were preserved in 20 mL RNAlater. Ten individuals from each triplicate experimental treatment were immediately transferred to 1 mL fresh RNAlater and stored at −20°C (**Figures 1A–C**).

Additionally, survival was estimated for copepods 24 h after exposure to 10 min salinity shock or handling stress with a control of non-handled individuals, as described for the initial survival estimation.

Statistical analysis and preparation of graphics were done using R (www.R-project.org, ver. 3.4.0). From the counts of dead and alive copepods, survival (%) was calculated for each exposure time and analyzed by linear regression. Differences between treatments were analyzed based on two-tailed *t*-tests of the regression coefficients (Sokal and Rohlf, 1995).

RNA Extraction, Library Preparation, and Sequencing

Total RNA was extracted from *A. tonsa* using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). For each treatment (control, handling stress, salinity shock), three biological replicates were analyzed, each consisting of 10 pooled individuals (prosome length = 758 ± 67 μm).

After removal of excess RNAlater (Sigma Aldrich, St. Louis, MO, USA), the copepods were homogenized in 50 μL RLT buffer using disposable micro-pestles, after which 550 μL RLT buffer was added. The samples were vortexed for 1–2 s, and then processed according to the manufacturer's protocol, with a final elution volume of 30 μL in RNase-free water.

RNA quality was assessed using an Agilent Tapestation 2200 with RNA High Sensitivity Assay (Agilent Technologies, Santa Clara, CA, USA). DNase treatment was not done, since previous extractions treated with the Turbo DNA-free kit (Ambion, Life Technologies, Carlsbad, CA, USA) exhibited significant degradation. The RNA quality profiling of *A. tonsa* exhibited a merged peak of 18S rRNA and 28S rRNA, presumably resulting from "hidden break" in 28S rRNA typical of many arthropods, which causes 28S rRNA to run at about the same size as 18S rRNA (McCarthy et al., 2015).

Library preparation was done using 360 ng total RNA from each sample following the manufacturer's protocol for the Illumina Stranded mRNA Library Preparation Kit (Illumina, Inc., San Diego, CA, USA). The libraries constructed from the 18 samples (3 treatments × 3 replicates × 2 incubation times) were multiplexed and sequenced in 2 runs across 4 lanes on the NextSeq500 platform (Illumina, San Diego, CA, USA), with a mid-output 150 cycle kit (FC-404-2001, Illumina, Inc., San Diego, CA, USA) with 75 bp paired-end reads and a sequencing depth of 25 million reads per sample. Library preparation and sequencing were carried out at the Center for Genome Innovation at the University of Connecticut (Storrs, CT, USA).

A reference transcriptome was determined from RNA extracted from a single individual of *A. tonsa* (female, prosome length 722 μm) selected at random from the control with 24 h of incubation. Total RNA (140 ng) was sequenced in 4 lanes on a NextSeq500 platform using a mid-output 300 cycles kit (FC-404-2003, Illumina, Inc., San Diego, CA, USA), with 150 bp paired-end reads resulting in ∼350 million reads.

De Novo Transcriptome Assembly and Differential Gene Expression Analysis

FastQC (ver. 0.7; Andrews, 2010) was used to validate the quality of the raw sequence reads. Illumina adapter sequences and low-quality reads (Phred score < 20) were removed using Trimmomatic (ver. 3; Bolger et al., 2014) in paired-end mode, with a sliding window across an average of 4 bases. Initial read biases, introduced by random hexamer priming under cDNA synthesis, were corrected by removing the first 12 bp of each read (Hansen et al., 2010). Reads > 50 bp after quality trimming were retained, resulting in a total of ∼225 million reads. The reference transcriptome was assembled *de novo* with Trinity (ver. 2.3.2; Grabherr et al., 2011) using default parameters for paired-end reads, with normalization to decrease run time and memory requirements.

The completeness of the reference transcriptome was evaluated using the Benchmarking Universal Single-Copy Orthologs (BUSCO, ver. 2; Simão et al., 2015), which defines a set of eukaryotic core genes to test the proportion and completeness of these genes in the transcriptome assembly. Bowtie2 (ver. 2.2.6; Langmead and Salzberg, 2012) was used to examine the RNA sequencing read representation of the assembly by realigning the input reads to the *de novo* transcriptome. Contig N50 and E90N50 statistics were computed based on the scripts

included in the Trinity software package, as well as transcript abundance estimation using Kallisto (ver. 0.43.0; Bray et al., 2016). The *A. tonsa* Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GFWY00000000. The version described in this paper is the first version, GFWY01000000.

The Trinotate annotation pipeline (ver. 3.0.2; Haas et al., 2013) was used to annotate the reference transcriptome using Swissprot (Bairoch and Apweiler, 1999); Pfam (Finn et al., 2010); eggNOG (Powell et al., 2012); KEGG (Kanehisa et al., 2012); and Gene Ontology (Ashburner et al., 2000).

The reads from the experimental samples were pseudoaligned (i.e., rapid determination of compatibility between reads and targets, without the need for alignment) to the reference transcriptome and quantified using Kallisto (ver. 0.43; Bray et al., 2016), with 100 rounds of bootstrapping. The bootstrapping option in Kallisto accounts for technical variability and is used to estimate the probability of correct assignment to a transcript. Differential gene (and transcript) analysis was performed with Sleuth (ver. 0.29; Pimentel et al., 2017), using the likelihood ratio test (LRT) and the Wald test in R Core Team (2017) to estimate significant results (ver. 3.4.0; R Core Team, 2017). Statistically significant (q-values < 0.05) differential gene expression is reported as beta values, which are bias estimators of the fold-change that accounts for the technical variability of transcripts and are reported as natural log values (Pimentel et al., 2017; see Supplementary Material S4 for results of Sleuth analysis of non-annotated transcripts).

The Trinity transcript identifications for non-annotated transcripts were added into the gene-level analysis in Sleuth. Transcripts showing statistically significant differential expression (q-value < 0.05) were isolated and annotation was attempted using Blast2Go and the RefSeq database with the arthropod taxonomy filter, in order to maximize the proportion of identified genes in the analysis (Götz et al., 2008) (Supplementary Material S2). Based on the BlastX results ($E \leq 10^{-3}$), gene symbols for identified transcripts were added to the gene-level analysis in Sleuth. Transcripts that could not be identified were excluded from the analysis of differential gene expression.

Functional enrichment of differentially expressed genes was performed using clusterProfiler (ver. 3.6.0; Yu et al., 2012) for gene ontologies (GO) of cell compartments (CC), biological processes (BP) and, molecular functions (MF). The genes annotated by Trinotate from the reference transcriptome were used as background gene list. Differentially up- and down-regulated genes for handling stress and salinity shock (15 min and 24 h after exposure) were used as input data. The functional enrichment was performed with a Fisher Exact test (p-value < 0.05, FDR < 0.1). Graphs and subsequent data handling was done in R Core Team (2017) using the ggplot2 package (ver. 2.2.1).

RESULTS

Survival

Survival of *A. tonsa* nauplii was statistically significantly ($p < 0.001$) affected by salinity shock (i.e., exposure to $S = 5$ for up to 10 min), in comparison to the control (**Figure 2E**, **Table 1E**). Naupliar survival in the control was 91 ± 4% (mean ± SD), while survival after salinity shock after 25 sec exposure was 95 ± 1%, declining to 22 ± 4% after 10 min exposure. Naupliar survival after handling stress was 97 ± 2% for exposure up to 10 min, which did not differ from the control, with an average survival of 97 ± 1% (**Figure 2D**, **Table 1D**). Survival for handling stress and salinity shock differed significantly by regression analysis ($p < 0.001$) (**Figure 2F**, **Table 1F**).

Adult individuals of *A. tonsa* were significantly affected both by salinity shock ($p < 0.001$) and handling stress ($p < 0.001$) for exposure up to 10 min (**Figures 1A,B**, **Tables 1A,B**) in comparison to the control, which exhibited survival of 98 ± 1% up to 10 min exposure time. Survival declined from 91 ± 2% after 25 s to 56 ± 7% after 10 min of exposure to handling stress. Survival declined in the salinity shock from 92 ± 1% after 25 s to 70 ± 5% after 10 min of exposure. Survival was higher when exposed to salinity shock than when exposed to handling stress, and a significant relation was found by regression analysis ($p < 0.05$, **Figure 2C**, **Table 1C**).

Survival of salinity shock and handling stress was estimated 24 h post 10 min of exposure. The control exhibited a survival of 99 ± 1%, salinity shock 47 ± 1% and handling stress 54 ± 2%. In the samples, with individuals exposed to handling stress, 34 ± 1% exhibited physical damage of the antennae, setae and antennules. Of the damaged individuals, 74 ± 3% were categorized as dead during staining. For the salinity shock samples, only 4 ± 3% exhibited damage, of which 53 ± 1% was categorized as dead.

De Novo Reference Transcriptome and Annotation

A total of ∼225 million reads >50 bp in length (after quality trimming) was retained for reference transcriptome assembly. The *de novo* assembled transcriptome consisted of 60,688 contiguous consensus sequences (contigs) grouped into 27,171 Trinity components ("genes") with a GC content of 38.49%. Statistics based on all transcript contigs had an N50 value of 1,874 bp, with an average contig length of 1,222.45 bp from for a total of 74,188,026 assembled bases (**Table 2**).

The quality of different Trinity transcriptome assemblies was evaluated using Bowtie2 for realignment of the reads to the reference, BUSCO evaluation of completeness, and E90N50 profiles of contig length (**Table 2**).

Of the reference input RNA sequencing reads realigned with Bowtie2, 90.35% were represented in the assembly of the chosen reference transcriptome (**Table 2**). The remaining unassembled reads, likely corresponded to low-expressed transcripts with insufficient coverage to enable assembly, was of low quality or resulted from aberrant reads.

Completeness of the transcriptome was examined by searching for single copy orthologs with 99.0% BUSCO scores. Results from 303 BUSCO groups searched were: single-copy: 44.9%; duplicated: 54.1%, fragmented: 0.7%; missing: 0.3% (**Table 2**).

The Ex90N50 transcript contig length of 2,731 bp was computed by combining Kallisto (ver. 0.43.0; Bray et al., 2016)

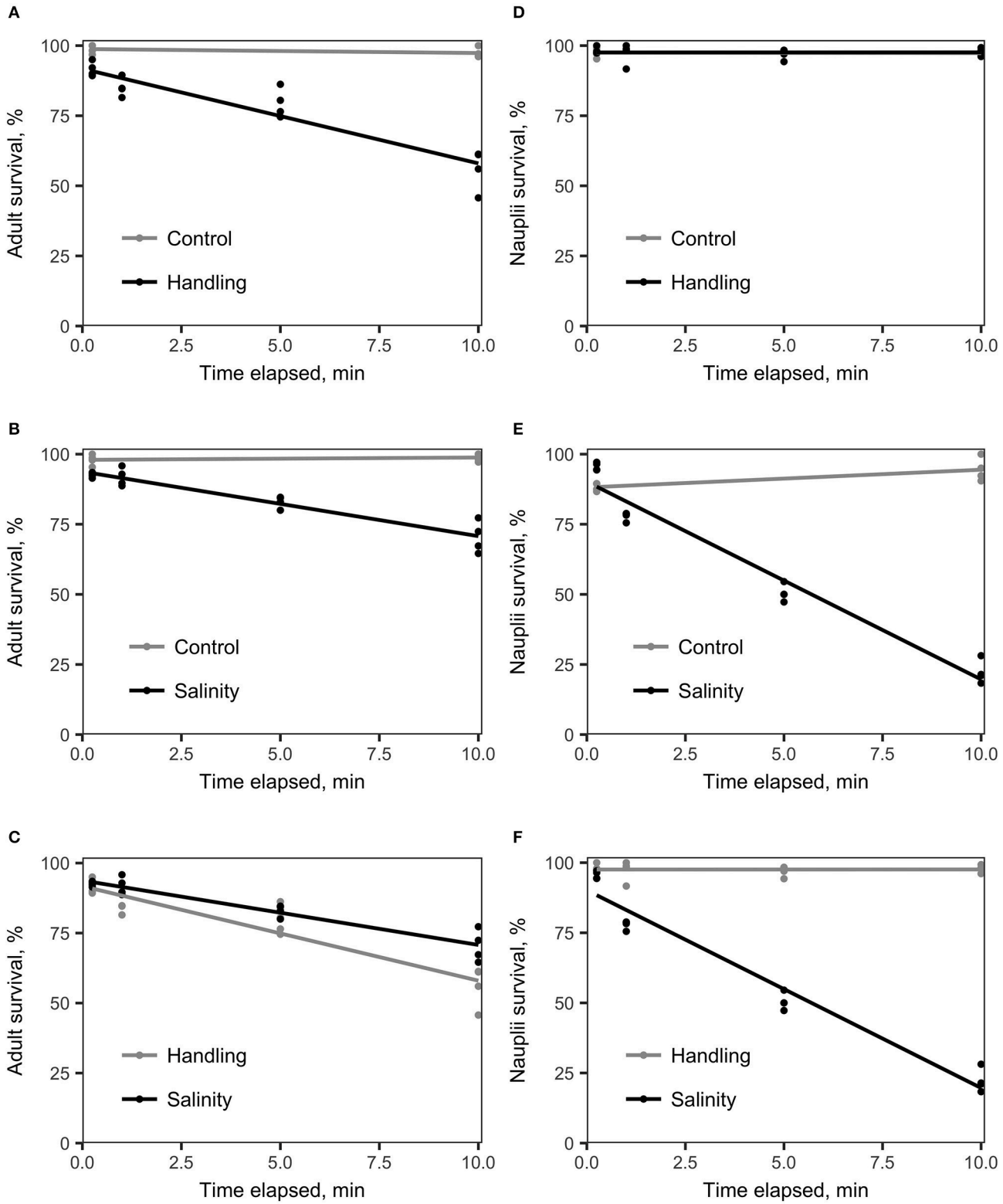

FIGURE 2 | Linear regression analysis of survival (%) vs. time elapsed for various treatments compared to the control treatment. The handling stress treatment consisted of placing copepods on a Nitex plankton net screen (adult mesh size: 250 μm; nauplii mesh size: 54 μm). The salinity shock treatment consisted of exposure of copepods to $S = 5$. **(A)** *A. tonsa* adults exposed to handling stress (black) vs. control (gray). The two treatments differed statistically significantly from

(Continued)

FIGURE 2 | each other (**Table 1**, **A**). **(B)** *A. tonsa* adults exposed to a salinity shock (black) vs. the control (gray). Survival in the two treatments differed statistically significantly (**Table 1**, **B**). **(C)** *A. tonsa* adults exposed to salinity shock (black) vs. individuals exposed to handling stress (gray). Survival in the two treatments differed statistically significantly from each other (**Table 1**, **C**). **(D)** *A. tonsa* nauplii exposed to handling stress (black) vs. control (gray). Survival in the two treatments did not differ statistically significantly from each other (see **Table 1**, **D**). **(E)** *A. tonsa* nauplii exposed the salinity shock (black), vs. control (gray). The two treatments differed statistically significantly from each other (**Table 1**, **E**). **(F)** *A. tonsa* nauplii exposed to the salinity shock (black) vs. individuals exposed to handling stress (gray). The two treatments differed statistically significantly from each other (**Table 1**, **F**).

TABLE 1 | *T*-test comparison of regression coefficients for linear regressions shown in **Figure 2**.

Graph		Treatment	Equation	R^2	SE_{slope}	*N*	SSE (b1-b2)	*T*-test	*df*	*p*
Adults	***A***	*Control (handling stress)*	98.78 − 0.14x	0.18	0.123	8	0.380	8.5042	20	<0.001
		Handling stress	91.75 − 3.38x	0.86	0.359	16				
	B	*Control (salinity shock)*	97.97 − 0.08x	0.07	0.124	8	0.243	9.8029	20	<0.001
		Salinity shock	93.77 − 2.30x	0.90	0.209	16				
	C	*Handling stress*	91.75 − 3.375x	0.86	0.359	16	0.416	−2.5815	28	<0.05
		Salinity shock	93.77 − 2.30x	0.90	0.209	16				
Nauplii	***D***	*Control (handling stress)*	97.48 - 0.02x	0.01	0.099	8	0.173	−0.1197	20	Not significant different
		Handling stress	97.56 + 0.01x	2.8E-05	0.141	16				
	E	*Control (salinity shock)*	88.12 + 0.63x	0.57	0.225	8	0.452	17.0094	20	<0.001
		Salinity shock	90.17 − 7.05x	0.96	0.392	16				
	F	*Handling stress*	97.56 +0.003x	2.8E-05	0.141	16	0.417	16.9311	28	<0.001
		Salinity shock	90.18 − 7.05x	0.96	0.392	16				

(A) *Adults, negative control vs. handling stress treatment;* ***(B)*** *Adults, control vs. salinity shock treatment;* ***(C)*** *Adults, handling stress vs. salinity shock;* ***(D)*** *Nauplii, control vs. handling stress treatment;* ***(E)*** *Nauplii, control vs. salinity shock;* ***(F)*** *Nauplii, handling stress vs. salinity shock.*
Slope corresponds to the regression coefficients of the linear regressions shown in ***Figure 2****.* SE_{slope} *is the standard error of the given regression coefficients. N is the sample size. SSE is the error sum of squares. T is the calculated "T-test" value and df is the degree of freedom where p is the probability value.*

and the ExN50 statistic script included in the Trinity package. Since N50 statistics discard read coverage, E90N50 gave an indication of whether deeper sequencing would result in higher quality assembly. The ExN50 profile peaked at N69, with a contig length of 3,075 bp (**Table 2**).

Considering the overall realignment, BUSCO profile, and ExN50 profile, we evaluated the reference transcriptome to be of acceptable quality for the differential gene expression analysis. The Trinotate annotation pipeline resulted in identification of 45% of the assembled Trinity transcripts (**Table 2**). The remaining 55% of unidentified transcripts were excluded from the differential gene expression analysis, after ensuring that the significantly differentially expressed transcripts could not be identified in any way (see Supplementary Material S4 for Sleuth analysis including unidentified transcripts.). The average pseudo-alignment of the experimental sample reads to the reference transcriptome using Kallisto was 82.2 ± 2.6% (mean ± SD) (**Table 2**).

From the Trinotate annotation, 80,047 Gene Ontologies (GO) and 24,463 Kyoto Encyclopedia of Genes and Genomes (KEGG) ontology (KO) terms were associated with the genes. It should be noted that multiple GO-terms could be assigned to the same gene. The GO-terms consisted ($p < 0.05$, FDR < 0.2) of 48% Biological Processes (BP), 30% Cellular Compartments (CC) and 22% Molecular Functions (MF) (see Supplementary Material S5 for all enriched GO terms).

Differentially Expressed Genes

The patterns of differential gene expression for copepods from the handling stress, salinity shock and control 15 min after exposure were not distinct in the Principal Component Analysis (PCA, **Figure 3A**). The control exhibited more distinct clustering from handling stress and salinity shock treatments 24 h after exposure (**Figure 3B**), suggesting that time following exposure has a significant effect on gene expression.

Two differentially expressed genes (DEGs; q-value < 0.05), one up- and one down-regulated, were identified 15 min after exposure to handling stress (**Figures 4**, **5**, **Table 3**; see Supplementary Material S1 for *b*-values; https://figshare.com/articles/S1a_Trinotate_Annotation_A_tonsa_xls/5928799/1).
The up-regulated DEG (*IPPK*, *Inositol-pentakisphosphate 2-* had two enriched BPs: Melanosome transport and determination of left/right symmetry (**Table 4**); two CCs: Ciliary basal body and Centrosome (**Table 4**); one MF: Inositol pentakisphosphate 2-kinase activity (**Table 4**); and two KOs: Phosphatidylinositol signaling system and Inositol phosphate metabolism (**Table 4**). The down-regulated DEG (*SIDT1*, *SID1 transmembrane family member 1*) was enriched in dsRNA transport (BP, **Table 4**) and RNA trans-membrane transporter activity (MF, **Table 4**).

Handling stress resulted in 276 DEGs 24 h after exposure, of which 177 were up- and 99 down-regulated (**Figures 4**, **6**, **Table 3**). None of the same DEGs were overlapping 15 min and

TABLE 2 | Assembly, validation, annotation and pseudo-alignment statistics.

ASSEMBLY					
#Raw reads	354,098,566				
#Reads after QC	225,217,658				
#Trinity contigs	60,662				
#Trinity components	27,171				
N50	1,874 bp				
Median contig length	790 bp				
Average contig length	1,222 bp				
VALIDATION					
E90N50	2,731 bp				
E69N50	3,075 bp				
Realignment with Bowtie2	90.35%				
BUSCO analysis	Completeness:	Single-copy:	Duplicated:	Fragmented:	Missing:
	99.0%	44.9%	54.1%	0.7%	0.3%
ANNOTATION					
Trinotate annotation	45%				
Blast2Go annotation	163 non-Trinotate-annotated differential expressed transcripts				
PSEUDO-ALIGNMENT					
Kallisto pseudo-alignment	82.2 ± 2.6%				

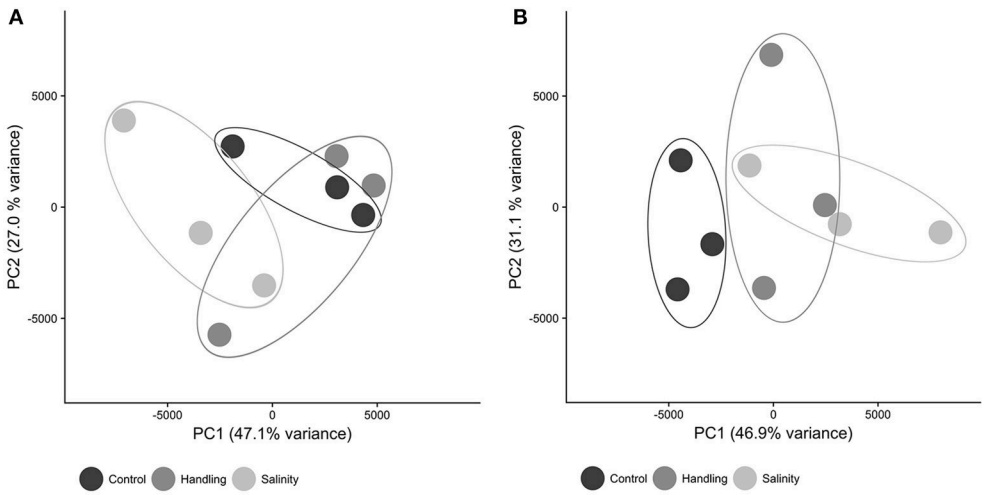

FIGURE 3 | Principal component analysis (PCA) dimensions 1 and 2 of expression values for all contigs in each sample. Symbols are: control (black), handling stress (dark gray); salinity shock (light gray). **(A)** After 10 min; **(B)** After 24 h. PCA was computed in R using the *Sleuth* package.

24 h after exposure. The salinity shock resulted in 7 DEGs (6 up- and 1 down-regulated) 15 min after exposure, which increased to 396 DEGs (221 up- and 175 down-regulated) 24 h after exposure (**Figure 4**, **Table 3**).

Among the up-regulated DEGs 15 min after exposure to salinity shock, the majority of enriched GO-terms were transport mechanisms, especially related to ER homeostasis and proteins (see Supplementary Material S5 for the full list, **Table 4** for top 10 of the GO-terms with most involved genes). The remainder of the enriched GO-terms were related to metabolic, homeostatic, and developmental processes (**Table 4**, Supplementary Material S5). The majority of these processes took place in the lysosomes (**Table 4**, Supplementary Material S5), Golgi apparatus (**Table 4**, Supplementary Material S5), and vesicles (**Table 4**, Supplementary Material S5). The three enriched KO-terms were related to signaling pathways and vitamin digestion and absorption (**Table 4**).

Two up-regulated DEGs (*ORNT1*, *Ornithine Transporter 1*; *MYH*, *Myosin Heavy chain*; Supplementary Material S1; https://figshare.com/articles/S1a_Trinotate_Annotation_A_tonsa_xls/5928799/1, **Table 5**) were overlapping 15 min and 24 h after exposure to salinity shock. ORNT was enriched in mitochondrial ornithine transport (BP, **Table 4**) taking place in the mitochondrial inner membrane (CC, **Table 4**), and related to two enriched MFs: L-ornithine transmembrane transporter activity and thiol-dependent ubiquitinyl hydrolase activity (**Table 4**).

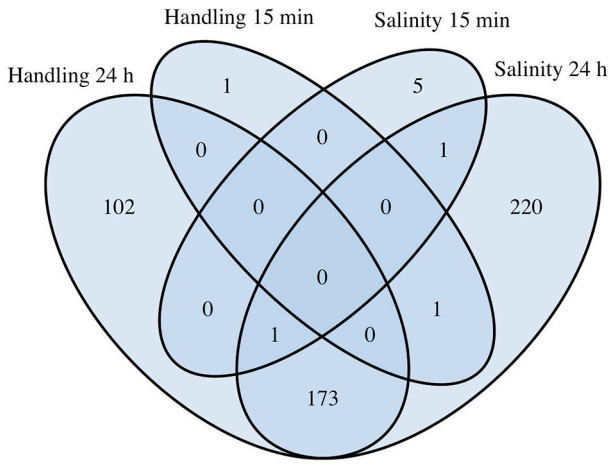

FIGURE 4 | Comparative analysis of differentially expressed genes (DEGs) in *Acartia tonsa* exposed to 10 min handling stress or 10 min salinity shock after 15 min or 24 h. The Venn diagram was constructed from overlapping transcripts in R with the library VennDiagram (ver. 1.6.17).

The overlap between handling stress and salinity shock 24 h after exposure included 174 DEGs. For the overlapping 112 up-regulated DEGs, 223 GO-terms were enriched (see Supplementary Material S5 for the full list, **Table 5** for top 10 of the GO-terms with most involved genes). No KO-terms were enriched. For the 62 down-regulated DEGs, 176 GO-, and 7 KEGG terms were enriched (Supplementary Material Table 5, **Table 5**).

The remaining non-overlapping 102 and 202 DEGs for handling stress and salinity shock 24 h post exposure, respectively, may be stressor-specific see Supplementary Material S5 for the full list, **Table 6** for top 10 of the GO-terms with most involved genes). For the 65 up-regulated non-overlapping DEGs 24 h post exposure to handling, 151 GO and 3 KO-terms were enriched. The 37 down-regulated DEGs had 94 enriched GO and 12 KO terms (Supplementary Material S5, **Table 6**). For non-overlapping DEGs 24 h post exposure to salinity shock, 109 were up- and 113 were down-regulated. The up-regulated DEGs resulted in the enrichment of 173 GO and 8 KO terms, while the down-regulated DEGs resulted in 226 GO and 2 KO terms (Supplementary Material S5, **Table 6**).

Inclusion of the Trinity transcript identifications in the Sleuth analysis (Supplementary Material S4) resulted in 350 DEGs (244 up- and 106 down-regulated) for the handling stress treatment after 24 h. The salinity shock resulted in 573 annotated DEGs (376 up- and 197 down-regulated) 24 h after exposure.

DISCUSSION

The results from our examination of transcriptome-wide responses and survival in relation to handling stress and salinity shock in *A. tonsa* clearly indicate that handling stress is a significant factor for experimental manipulation of this species. Failure to consider this parameter in, for instance, biomarker-related transcriptional studies is likely to lead to inaccurate interpretation of the stressors' impact.

The survival rates of the exposed nauplii were not affected by handling stress. This may be because of their small size and lack of fragile appendages, allowing a boundary layer of seawater to form around them as protection from physical interaction with the plankton net. Since handling stress did not negatively impact naupliar survival, we used only adult individuals of *A. tonsa* for the definitive experiments for RNA sequencing analysis. The use of adult females is most usual for copepod incubation experiments to obtain physiological rates (e.g., oxygen consumption, grazing, growth, and specific egg production) based on published results.

Adult copepod survival decreased by 44 ± 7% (mean ± SD) after 10 min exposure to handling stress. The average mortality reported by Jepsen et al. (2007) was 17 ± 1% per day, which was considered to result from handling stress in adult individuals of *A. tonsa* every 12 h during their study. In comparison, other studies have reported constant daily mortality ranging from 5 to 10% (Medina and Barata, 2004; Drillet et al., 2014; Nilsson et al., 2017). The observed handling stress effect on survival in the present study is considered very high and indicative of impacts of handling stress for adult individuals of *A. tonsa*. Handling of adult copepods should therefore be done carefully—and quickly—in order to minimize the imposed stress, in contrast to nauplii, which do not seem to be affected on survival.

In comparison to handling stress, salinity shock resulted in a slightly higher adult survival (29 ± 5%) after 10 min of exposure. Calliari et al. (2008) found that an abrupt reduction in salinity from $S = 35$ to $S = 4$ resulted in 31% decrease in survival, which is comparable to the decrease of 22–35% observed in the present study.

Survival 24 h after exposure to 10 min handling stress or salinity shock declined 45 ± 1.7% and 51 ± 1.6%, respectively. For handling stress, this was an additional decline of ∼7% compared to 15 min post exposure. For the salinity shock, the post-exposure period of 24 h resulted in higher mortality than for handling stress, with an additional decline in survival of ∼22%. This suggests that handling stress results in higher instant mortality than salinity shock, while salinity shock is more lethal over an extended post-exposure period. Part of the instant mortality may be due to the physical damages (i.e., broken antennae) observed for about one-third of the individuals, most of which were categorized as dead. In addition to the elevated mortality, the higher number of DEGs suggests that salinity shock is more stressful than handling.

In order to replicate the impact of "collection stress" and laboratory handling stress on *A. tonsa*, it was important to establish a baseline for copepods that were not exposed to handling stress. Since this is the first study examining impacts of handling stress at the transcriptome-wide level, there is no available information on the time course of the copepods' responses after handling stress. To avoid this as a bias in our experiments, we cultured *A. tonsa* from eggs to the desired life-stages in ideal culture conditions without disturbing the copepods.

For field studies, it is often impossible to know how long the copepods may have been in contact with a plankton net during collection and before preservation (e.g., Mack et al., 2012). According to the field guide by Goswami (2004), it is

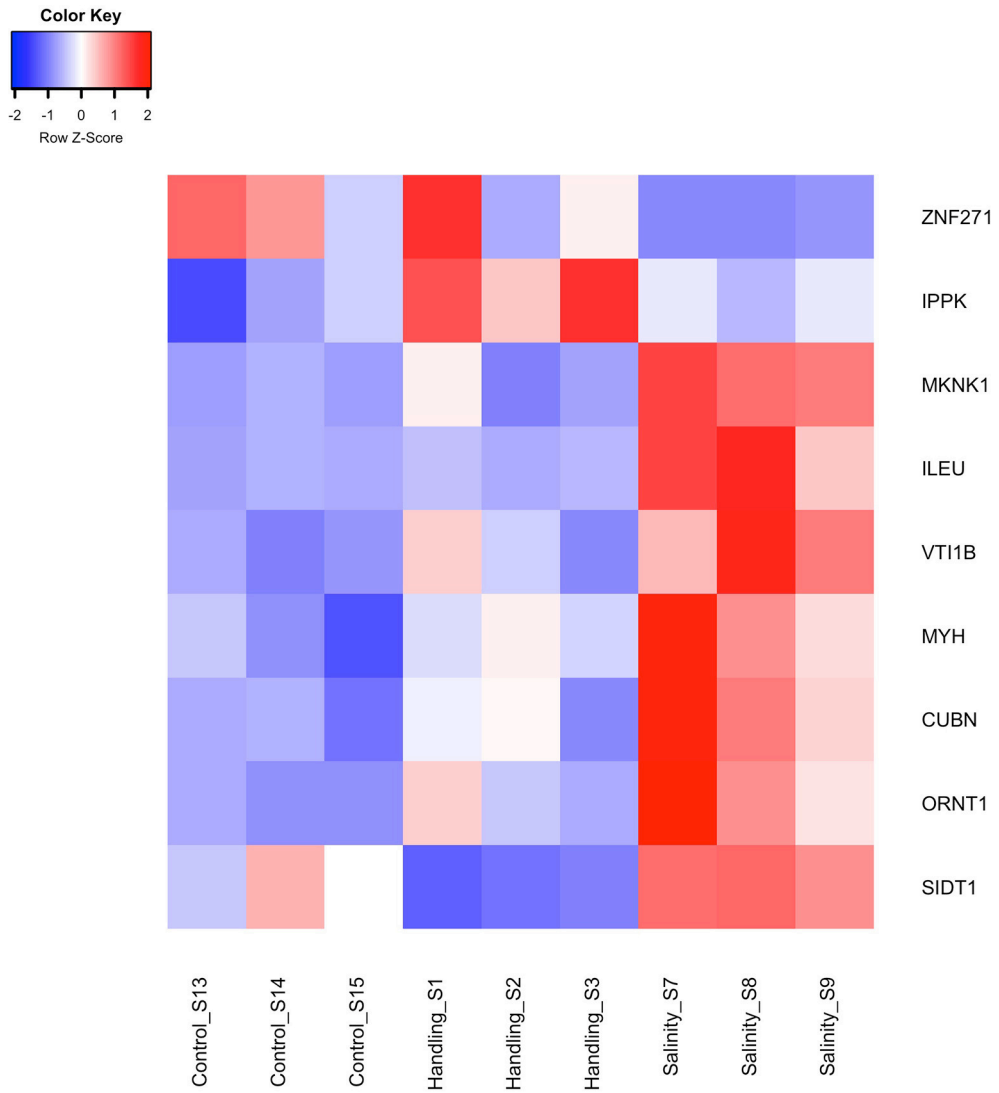

FIGURE 5 | Heat map of differentially expressed genes ($q < 0.05$) for the handling stress and salinity shock treatments 15 min post exposure relative to the control. The color scale of transcript expressions is normalized as z-scores; up-regulated transcripts are red and down-regulated are blue. The heat map was generated in R using the library *gplots* (version 3.0.1).

recommended to collect zooplankton by towing a plankton net at slow speed (1.5–2.0 knots) for 5–10 min. Additional collection time is used in recovering the plankton net and handling the copepods for different experimental purposes. The mortality of field-collected copepods, however, has been shown to range from 0 to 90% based on direct observation and 13–37% based on neutral red staining (Elliott and Tang, 2009). This is within the range of the survival decrease observed in the present study (44 ± 7%) and suggests that observed mortality in field-collected copepods may be the result of handling stress. For laboratory studies, copepods are usually exposed to plankton net screens for shorter periods. But often other tools used for transferring copepods, such as pipettes and tweezers, may also result in physical damage and stress.

Based on the observed mortalities, we chose the exposure time of 10 min for each stressor, which is within the range of handling time described in published studies, although sufficient to ensure that we would induce a transcriptional response (Elliott and Tang, 2009; Mack et al., 2012).

An additional source of uncertainty regarding responses at the transcriptional level was the impact of the length of time post-exposure before preservation for analysis.

We found only two (1 up- and 1 down-regulated) and seven (6 up- and 1 down-regulated) DEGs 15 min after exposure to handling stress and salinity shock, respectively (**Figure 5**, **Table 3**). None of these DEGs were in common between the treatments or have previously been used as transcriptional biomarkers. Handling stress resulted in 276 DEGs and salinity

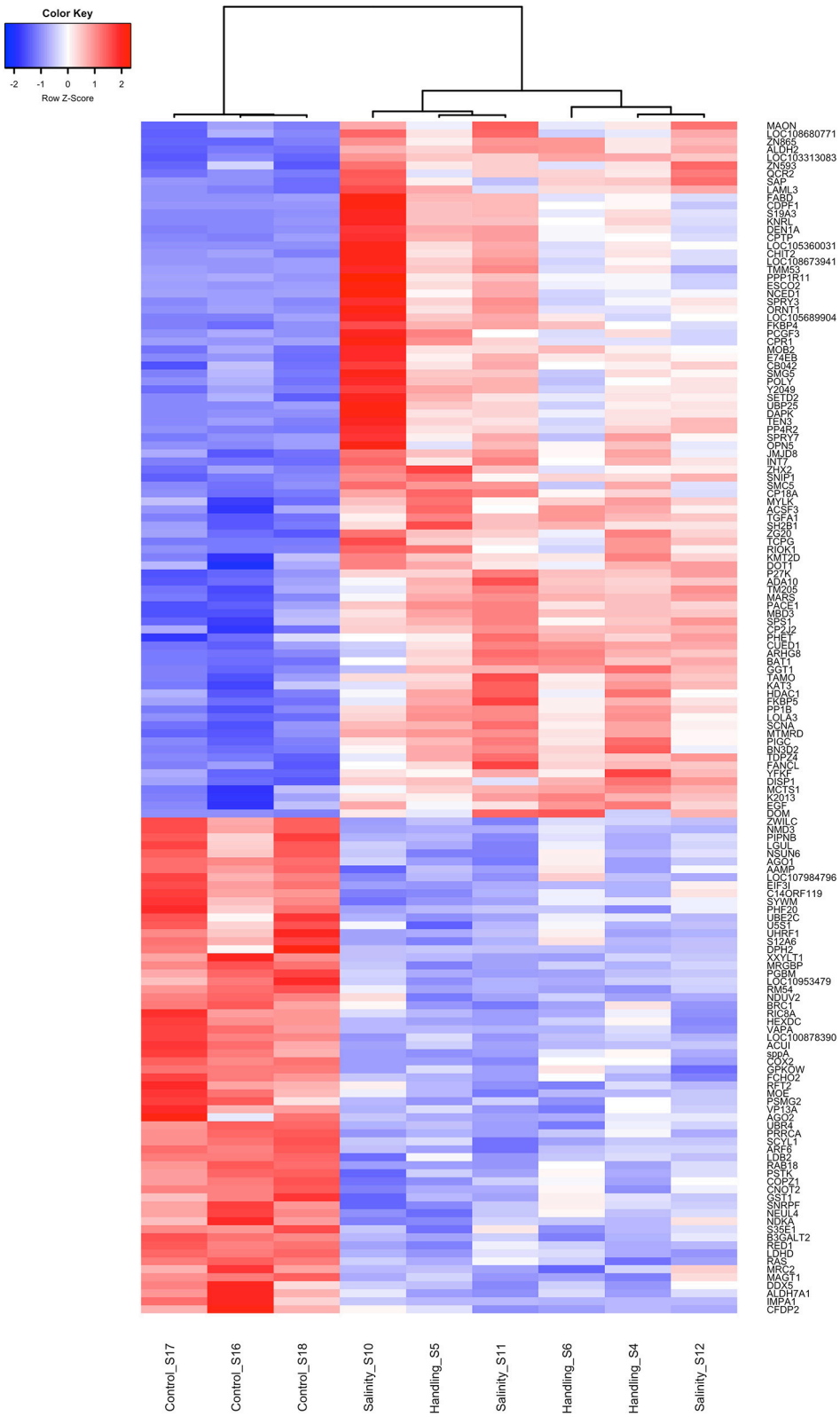

FIGURE 6 | Heat map of overlapping differentially expressed genes ($q < 0.05$) for the handling stress and salinity shock treatments 24 h post exposure relative to the control. Similarities between samples are shown as a dendrogram, with hierarchical clustering based on Pearson correlation with complete distance determination. The color scale of transcript expressions is normalized as z-scores; up-regulated transcripts are red and down-regulated are blue. The heat map was generated in R using the library *gplots* (version 3.0.1).

TABLE 3 | Overview of the number (#) of differentially expressed genes (DEGs).

# DEGs for following treatments:		Handling	Salinity shock
15 min	Total	2	7
	Up-regulated	1	6
	Down-regulated	1	1
	Treatment specific	2	7
24 h	Total	276	396
	Up-regulated	177	221
	Down-regulated	99	175
	Treatment specific	102	222
# Overlapping DEGs			
Handling stress vs. salinity shock, 15 min		0	
Handling stress vs. salinity shock, 24 h		174	
Handling stress, 15 min vs. 24 h		0	
Salinity shock, 15 min vs. 24 h		2	

shock in 396 DEGs 24 h after exposure. Of these, 174 DEGs (112 up- and 62 down-regulated) were overlapping. The up-regulated expression of these genes may provide general protection against multiple stressors. However, the time period following exposure clearly had significant impact on whether there was a detectable and measureable stress response.

The one up-regulated DEG 15 min after exposure to handling, *Inositol-Pentakisphosphate 2-Kinase* (*IPPK*), was assigned two BPs (melanosome transport and determination of left/right symmetry). *IPPK* in yeast is involved in the transcriptional regulation of responses to environmental and nutritional changes; in plants, it is involved in stress signaling, and in mouse embryonic development (e.g., Tsui and York, 2010). The role of *IPPK* is thus very diverse among species and therefore the enriched BPs seems difficult to explain. In *A. tonsa*, the early up-regulation of *IPPK* may indicate its role in initiating a transcriptional response to handling stress.

The gene product of the down-regulated *SID1 Transmembrane Family Member 1* (*SIDT1*) is involved in RNA-interference (RNAi), by transporting dsRNA across cellular membranes (e.g., Whangbo et al., 2017). The down-regulation suggests that gene silencing of RNAi inhibited genes are being removed, which allows transcription.

The majority of enriched GO terms for up-regulated DEGs 15 min post exposure to salinity were related to protein transport, Endoplasmic Reticulum (ER), and protein homeostasis. This is an indication of ER stress, which typically induces the unfolded protein response (UPR) in order to improve the imbalance between protein load and folding capacity of the ER (Hori et al., 2006; Hetz and Papa, 2017).

Enrichment of GO terms related to metabolic processes indicated the need for cellular energy and is in agreement with the observation by Calliari et al. (2006) that *A. tonsa* modulates its energy balance in relation to salinity stress. The enrichment of vitamin digestion and absorption (KO, **Table 4**) for *MAP kinase-interacting serine/threonine protein kinase 2* (*MAPK*) could reflect a need for energy. However, *MAPK* is also induced in response to

TABLE 4 | Enriched gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes ontologies (KEGG, KO) for differentially expressed genes (DEGs) 15 min post exposure to handling-stress or salinity shock.

Description	Cat.	*p*	Change	ID	Genes
15 min post exposure to 10 min handling-stress (2 DEGs in total)					
Inositol pentakisphosphate 2-kinase activity	MF	0.0001	Up	GO:0035299	*IPPK*
Melanosome transport	BP	0.007	Up	GO:0032402	*IPPK*
Determination of left/right symmetry	BP	0.003	Up	GO:0007368	*IPPK*
Ciliary basal body	CC	0.004	Up	GO:0036064	*IPPK*
Centrosome	CC	0.02	Up	GO:0005813	*IPPK*
RNA transmembrane transporter activity	MF	0.0001	Down	GO:0051033	*SIDT1*
dsRNA transport	BP	0.001	Down	GO:0033227	*SIDT1*
Phosphatidylinositol signaling system	KEGG	0.005	Up	KO:04070	*IPPK*
Inositol phosphate metabolism	KEGG	0.006	Up	KO:00562	*IPPK*
15 min post exposure to 10 min salinity shock (7 DEGs in total)					
Lysosomal membrane	CC	0.002	Up	GO:0005765	*VTI1B/CUBN*
Golgi apparatus	CC	0.01	Up	GO:0005794	*VTI1B/CUBN*
L-ornithine transmembrane transporter activity	MF	0.0007	Up	GO:0000064	*ORNT1*
Mitochondrial ornithine transport	BP	0.0007	Up	GO:0000066	*ORNT1*
Cobalamin transporter activity	MF	0.0007	Up	GO:0015235	*CUBN*
Extrinsic component of external side of plasma membrane	CC	0.0007	Up	GO:0031232	*CUBN*
Cobalamin transport	BP	0.001	Up	GO:0015889	*CUBN*
Hemoglobin import	BP	0.001	Up	GO:0020028	*CUBN*
Hemoglobin binding	MF	0.001	Up	GO:0030492	*CUBN*
Endocytic vesicle membrane	CC	0.001		GO:0030666	*CUBN*
Vitamin digestion and absorption	KEGG	0.006	Up	KO:04977	*MKNK2*
HIF-1 signaling pathway	KEGG	0.02	Up	KO:04066	*MKNK1*
Insulin signaling pathway	KEGG	0.02	Up	KO:04910	*MKNK1*

The top 10 (or fewer in some cases) GO-terms with most genes involved are shown for up- and down regulated DEGs for each treatment. The DEGs were sorted by the number of involved genes, and then by p-values for the most significant enrichments (p). Cat., ontology category; can be BP, Biological Process; CC, Cellular Compartment; MF, Molecular Function; or KEGG. p, false discovery rate corrected p-value (FDR < 0.2). Change, imply if gene expression were up— or down regulated in relation to the negative control. ID, ontology ID. Genes, abbreviations for DEGs; full names can be found in Supplementary Material S3. No DEGs 15 min post exposure were overlapping between handling-stress and salinity shock. Enrichment of GO and KEGG terms were done using the R-package ClusterProfiler (ver. 3.6.0, Yu et al., 2012) with the Trinotate annotated transcriptome as background gene list.

TABLE 5 | Enriched gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes ontologies (KEGG, KO) for overlapping differentially expressed genes (DEGs) 24 h post exposure to handling-stress and salinity shock, as well as overlapping DEGs 15 min and 24 h post exposure to salinity shock.

Description	Cat.	*p*	Change	ID	Genes
Overlapping DEGs, 24 h post exposure to handling stress and salinity shock (174 DEGs in total)					
Neurogenesis	BP	0.02	Up	GO:0022008	*HDAC1/PP4R2/DOM/LOLA3/SPS1*
Cellular response to DNA damage stimulus	BP	0.02	Up	GO:0006974	*UBP25/INT7/FANCL/BD1L1/SMC5*
Chromosome	CC	0.01	Up	GO:0005694	*ZG20/INT7/SETD2/BD1L1*
Oogenesis	BP	0.02	Up	GO:0048477	*HDAC1/DOM/E74EB/BRN*
Ubiquitin protein ligase binding	MF	0.04	Up	GO:0031625	*SMG5/FANCL/TDPZ4/TS101*
Response to estradiol	BP	0.003	Up	GO:0032355	*MBD3/GGT1/ALDH2*
Chaperone-mediated protein folding	BP	0.003	Up	GO:0061077	*TCPG/FKBP4/FKBP5*
mRNA export from nucleus	BP	0.02	Up	GO:0006406	*SMG5/NU155/NUP62*
Chromatin	CC	0.02	Up	GO:0000785	*ESCO2/HDAC1/MBD3*
Transcription corepressor activity	MF	0.02	Up	GO:0003714	*ZHX2/HDAC1/TS101*
Plasma membrane	CC	0.006	Down	GO:0005886	*SYWM/LGUL/CNOT2/FCHO2/AAMP/S12A6/NDKA/ARF6/MAGT1/RIC8A/RAS/UBR4/PGBM*
Regulation of transcription from RNA polymerase II promoter	BP	0.0003	Down	GO:0006357	*MRGBP/BRC1/LGUL/LDB2/CNOT2/DDX5*
Positive regulation of transcription from RNA polymerase II promoter	BP	0.02	Down	GO:0045944	*UHRF1/BRC1/AGO2/PHF20/DDX5/AGO1*
GTP binding	MF	0.007	Down	GO:0005525	*RAB18/U5S1/NDKA/ARF6/RAS*
Double-stranded RNA binding	MF	0.0004	Down	GO:0003725	*RED1/AGO2/AGO1*
RNA secondary structure unwinding	BP	0.002	Down	GO:0010501	*AGO2/DDX5/AGO1*
Extracellular matrix	CC	0.003	Down	GO:0031012	*U5S1/DDX5/PGBM*
Angiogenesis	BP	0.004	Down	GO:0001525	*AAMP/S12A6/PGBM*
Intracellular ribonucleoprotein complex	CC	0.007	Down	GO:0030529	*AGO2/DDX5/AGO1*
Brain development	BP	0.008	Down	GO:0007420	*RED1/RAB18/PGBM*
Aminoacyl-tRNA biosynthesis	KEGG	0.006	Down	KO:00970	*SYWM/PSTK*
Pyruvate metabolism	KEGG	0.01	Down	KO:00620	*LGUL/LDHD*
Spliceosome	KEGG	0.02	Down	KO:03040	*U5S1/DDX5*
RNA transport	KEGG	0.03	Down	KO:03013	*NMD3/EIF3I*
Other glycan degradation	KEGG	0.03	Down	KO:00511	*HEXDC*
Proteoglycans in cancer	KEGG	0.04	Down	KO:05205	*PGBM/DDX5*
Vitamin digestion and absorption	KEGG	0.04	Down	KO:04977	*RFT2*
Overlapping DEGs, 15 min and 24 h post exposure to salinity shock (2 DEGs in total)					
Mitochondrial ornithine transport	BP	0.0002	Up	GO:0000066	*ORNT1*
Mitochondrial inner membrane	CC	0.03	Up	GO:0005743	*ORNT1*
L-ornithine transmembrane transporter activity	MF	0.0002	Up	GO:0000064	*ORNT1*
Thiol-dependent ubiquitinyl hydrolase activity	MF	0.002	Up	GO:0036459	*ORNT1*

The top 10 (or fewer in some cases) GO-terms with most genes involved are shown for up- and down-regulated DEGs. The DEGs were sorted by number of involved genes, and then by p-values for the most significant enrichments (p). Cat., ontology category; can be BP, Biological Process; CC, Cellular Compartment; MF, Molecular Function; or KEGG. p is the false discovery rate corrected p-value (FDR < 0.2). Change, imply if gene expression was up—or down regulated in relation to the negative control; ID, ontology ID; Genes, abbreviations for DEGs; full names can be found in Supplementary Material S3. None of the DEGs 15 min post exposure is overlapping between handling-stress and salinity shock. Enrichment of GO and KEGG terms were done using the R-package ClusterProfiler (ver. 3.6.0, Yu et al., 2012) with the Trinotate annotated transcriptome as background gene list.

environmental stress as a part of a signaling cascade, hence the two signaling pathways in **Table 4** (Waskiewicz, 1997).

The KEGG enrichment was done using general KEGG Ontology (KO) terms, which are related to the usual model organisms, human and mouse. The enriched terms and the actual functions may therefore differ in relation to copepods.

Five of the overlapping DEGs 24 h post exposure to handling stress and salinity shock were enriched for the BP, neurogenesis. These include *Histone deacetylase 1* (*HIDAC1*),

TABLE 6 | Enriched gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes ontologies (KEGG, KO) for non-overlapping differential expressed genes (DEGs) 24 h post exposure to handling-stress and salinity shock.

Description	Cat.	*p*	Change	ID	Genes
24 h post exposure to handling-stress (102 non-overlapping DEGs in total)					
ATP binding	MF	0.002	Up	GO:0005524	*PTPA/CDK8/ULK3/TRIO/PRS6B/SKIV2/CDK12/MRP2/PRP16/CDC48/MOS/CNNM2/PRPS1/MAST1*
RNA polymerase II carboxy-terminal domain kinase activity	MF	0.001	Up	GO:0008353	*CDK8/CDK12*
Negative regulation of TOR signaling	BP	0.004	Up	GO:0032007	*FLCN/UBR2*
Cellular amino acid metabolic process	BP	0.005	Up	GO:0006520	*DDC/KBL*
Cyclin-dependent protein serine/threonine kinase activity	MF	0.006	Up	GO:0004693	*CDK8/CDK12*
Guanyl-nucleotide exchange factor activity	MF	0.03	Up	GO:0005085	*FLCN/TRIO*
Cytoskeleton organization	BP	0.03	Up	GO:0007010	*RHO1/MAST1*
Fibrillar center	CC	0.03	Up	GO:0001650	*ZFP58/CDK12*
Pyridoxal phosphate binding	MF	0.03	Up	GO:0030170	*DDC/KBL*
Ribose phosphate diphosphokinase complex	CC	0.007	Up	GO:0002189	*PRPS1*
Extracellular region	CC	0.02	Down	GO:0005576	*PA21B/SNO1/APOD/EXGB*
Transporter activity	MF	0.001	Down	GO:0005215	*APOD/SYPL1/MYP2*
Cholesterol binding	MF	0.002	Down	GO:0015485	*APOD/MYP2*
Axoneme	CC	0.003	Down	GO:0005930	*GAS8/BBS1*
Ciliary basal body	CC	0.009	Down	GO:0036064	*GAS8/BBS1*
Lamellipodium	CC	0.02	Down	GO:0030027	*ARPC3/PTN13*
Brain development	BP	0.03	Down	GO:0007420	*APOD/GAS8*
Cleavage in ITS2	BP	0.004	Down	GO:0000448	*NOL9*
Glycerol dehydrogenase [NAD+] activity	MF	0.004	Down	GO:0008888	*ADH1*
Alkaloid metabolic process	BP	0.004	Down	GO:0009820	*SNO1*
mTOR signaling pathway	KEGG	0.001	Up	KO:04150	*ULK3/RHO1/FLCN*
Antifolate resistance	KEGG	0.001	Up	KO:01523	*MRP2/MRP2*
Sphingolipid signaling pathway	KEGG	0.007	Up	KO:04071	*RHO1/MRP2*
24 h post exposure to salinity shock (222 non-overlapping DEGs in total)					
Plasma membrane	CC	0.001	Up	GO:0005886	*SIAH1/PIM1/RBGPR/C2CD5/CADN/CDIPT/DPOE1/NID2/ATPB1/PRC1/LIMS2/TRPA1/IGF1R/MOT12/MFSD5/ENTP6/MYO1G/UNC5B/S12A9/JMJD6*
Integral component of membrane	CC	0.03	Up	GO:0016021	*CSMD1/SSRD/CADN/CDIPT/S22A7/UBAC2/P3/TRPA1/PEN2/TM2D1/IGF1R/XYLT/MOT12/MFSD5/ENTP6/UNC5B/CHSTE/NOX5/S12A9/PERT*
Calcium ion binding	MF	0.005	Up	GO:0005509	*C2CD5/CADN/NID2/CAB45/ESYT2/NOX5/PERT*
Identical protein binding	MF	0.02	Up	GO:0042802	*SIAH1/ASH1/RNF4/PRC1/ESYT2/CASC3*
Ubiquitin-protein transferase activity	MF	0.02	Up	GO:0004842	*SIAH1/RNF4/FBX30/BIRC3/TRIM2*
Apoptotic process	BP	0.04	Up	GO:0006915	*SIAH1/PIM1/UNC5B/NOX5/THOC6*
Cytokinesis	BP	0.002	Up	GO:0000910	*PRC1/KI13A/NOX5*
rRNA binding	MF	0.002	Up	GO:0019843	*RPF1/RS4X/RM16*
Cell surface receptor signaling pathway	BP	0.01	Up	GO:0007166	*TSN31/BIRC3/JMJD6*
Intracellular ribonucleoprotein complex	CC	0.02	Up	GO:0030529	*RS4X/CASC3/JMJD6*
Nucleus	CC	0.002	Down	GO:0005634	FOXG1/CLCA2/ZG17/ZFP28/NSF1C/DIDO1/PAK2/LARK/MCE1/SAP30/ZN207/NAA20/ZN317/METL4/ZN33B/TRI23/SQD/ABRU/ABHEB/TUT4/PSMD4/ZNF28/NUD16/HDAC3/CDK7/PLAG1/ASCC1/SNPC3/ATE1/RAD51/ZN155/AKR/SMCE1/ZN112/XCP1/ZFHX3/HSP70/PRD16/SRPK2/DMAD/SLF1/ADK/S18L2/SENP7

(Continued)

TABLE 6 | Continued

Description	Cat.	*p*	Change	ID	Genes
Nucleoplasm	CC	0.01	Down	GO:0005654	NSF1C/DIDO1/GIT2/DCA11/PSMD4/ZNF28/NUD16/PP6R3/S40A1/PLAG1/RAD51/AKR/SMCE1/ZN112/ZFHX3/GPN1/PRD16/SRPK2/RAD1
Transcription, DNA-template	BP	0.04	Down	GO:0006351	FOXG1/ZG17/ZFP28/DIDO1/SAP30/ZN317/ZN33B/ABRU/ZNF28/HDAC3/PLAG1/ASCC1/SNPC3/ZN155/AKR/ZN112/ZFHX3/PRD16/S18L2
Transcription factor activity, sequence-specific DNA binding	MF	0.04	Down	GO:0003700	FOXG1/ZFP28/ZN317/ZN33B/ABRU/ZNF28/PLAG1/ZN155/ZN112/PRD16
Golgi membrane	CC	0.03	Down	GO:0000139	*CHSTB/STX5/GTR1/TRI23/PP6R3/SEC20/GLT35*
Identical protein binding	MF	0.03	Down	GO:0042802	*GTR1/G6PD1/TRI23/PSMD4/NUD16/RAD51*
RNA polymerase II core promoter proximal region sequence-specific DNA binding	MF	0.02	Down	GO:0000978	*ZNF28/PLAG1/AKR/SMCE1*
Precatalytic spliceosome	CC	0.01	Down	GO:0071011	*LARK/SQD/HSP70*
Chromatin	CC	0.03	Down	GO:0000785	*SQD/WGE/RAD51*
Spindle	CC	0.04	Down	GO:0005819	*DIDO1/ZN207/PIN4*
Parkinson's disease	KEGG	0.004	Up	KO:05012	*UBB/QCR7/PPIA*
RNA transport	KEGG	0.005	Up	KO:03013	*THOC6/NUP85/CASC3*
Huntington's disease	KEGG	0.007	Up	KO:05016	*QCR7/PPIA/DCTN2*
Glycosaminoglycan biosynthesis - chondroitin sulfate/dermatan sulfate	KEGG	0.001	Up	KO:00532	*XYLT/CHSTE*
Acute myeloid leukemia	KEGG	0.009	Up	KO:05221	*PIM1/PANG1*
Bile secretion	KEGG	0.010	Up	KO:04976	*S22A7/ATPB1*
Cardiac muscle contraction	KEGG	0.011	Up	KO:04260	*QCR7/ATPB1*
Inositol phosphate metabolism	KEGG	0.015	Up	KO:00562	*YRBE/CDIPT*
Central carbon metabolism in cancer	KEGG	0.003	Down	KO:05230	*P55G/GTR1/G6PD1*
Thyroid hormone signaling pathway	KEGG	0.002	Down	KO:04919	*P55G/HDAC3/GTR1*

The top 10 (if there is 10) GO-terms with most genes involved are here shown for up- and down-regulated DEGs for each treatment. The DEGs (genes) sorted by number of involved genes, and then based on p-values with most significant enrichments (p). Cat., ontology category; can be BP, Biological Process; CC, Cellular Compartment; MF, Molecular Function, or KEGG. p is the false discovery rate corrected p-value (FDR < 0.2). Change, imply if gene expression was up – or down regulated in relation to the negative control; ID, ontology ID; Genes, abbreviations for DEGs; full names can be found in Supplementary Material S3. None of the DEGs 15 min post exposure is overlapping between handling-stress and salinity shock. Enrichment of GO and KEGG terms were done using the R-package ClusterProfiler (ver. 3.6.0, Yu et al., 2012) with the Trinotate annotated transcriptome as background gene list.

serine/threonine-protein phosphatase 4 regulatory subunit 2 (*PP4R2*), *helicase domino* (*DOM*), *longitudinals lacking protein* (*LOLA3*) and *selenide water dikinase* (*SPS1*). Even though they are enriched in the GO-term, neurogenesis, it is noteworthy that only *LOLA3* is directly linked to neurogenesis (e.g., Goeke et al., 2003). The gene product resulting from *HIDAC1* is mainly a regulator of gene expression for other genes responsible for histone de-acetylation (e.g., Kelly and Cowley, 2013). *DOM* is, like *HIDAC1,* also responsible for transcriptional regulation by chromatin remodeling (Sif, 2004). Especially *HIDAC1* is also enriched for terms related to chromatin remodeling, like chromatin (CC) and transcription corepressor activity (MF). *PP4R2* has functional roles in cell development, differentiation, apoptosis, tumor progression and DNA-repair (e.g., Shui et al., 2007; Nakada et al., 2008; Liu et al., 2012).

In addition to being enriched in GO-terms related to transcriptional regulation, oogenesis is enriched for *HDAC1*, *DOM*, *Ecdysone-induced protein 74EF isoform B* (*E74EB*) and *beta-1,3-galactosyltransferase BRN (BRN)*. Both *E74EF* and *BRN* are involved in oogenesis (Goode et al., 1996; Paul et al., 2005). When exposed to stressful conditions, energy of an individual tends to be reallocated from fecundity and growth to survival mechanisms (López-Maury et al., 2008; de Nadal et al., 2011). Thus, egg production of copepods decreases when the surrounding environment is sub-optimal (Calliari et al., 2006; Peck and Holste, 2006). The up-regulation of oogenesis may indicate that homeostasis in *A. tonsa* has been restored to such extent that there is energy for egg production.

Even though fecundity-related mechanisms were enriched for up-regulated overlapping DEGs 24 h post exposure to handling stress and salinity shock, stress related mechanisms were also present among the enriched GO-terms (**Table 5**, Supplementary Material S5). This includes cellular response to DNA damage stimulus, ubiquitin protein ligase binding and chaperone-mediated protein folding (**Table 5**).

Heat shock proteins (*hsps*), especially *heat shock protein 70 kDa* (*hsp70*), have been frequently used as a transcriptional indicator of stress in copepods (Voznesensky et al., 2004; Tartarotti and Torres, 2009; Lauritano et al., 2011, 2016; Nilsson et al., 2013; Chan et al., 2014; Petkeviciute et al., 2015; Aguilera et al., 2016;

Smolina et al., 2016; Rahlff et al., 2017). We found significant, but small, down-regulation of *hsp70* in response to salinity shock 24 h after exposure (Supplementary Material S1; https://figshare.com/articles/S1a_Trinotate_Annotation_A_tonsa_xls/5928799/1). *Heat shock cognate 71 kDa* (*hsc70*), which is a member of the hsp70 family, was slightly up-regulated for handling stress and salinity shock 24 h after exposure (Supplementary Material S1; https://figshare.com/articles/S1a_Trinotate_Annotation_A_tonsa_xls/5928799/1). Aruda et al. (2011) found that the heat shock proteins, *hsp70a*, *hsp21,* and *hsp22*, had significantly higher expression 3 h after handling with a plankton net, but did not find any significant differences 2 h after exposure. Rahlff et al. (2017) found significant changes in expression of *hsp70* in response to handling, which was reduced to negligible levels after 24 h. These prior studies suggest that the expression of *hsp70* peaks within 24 h after stress exposure, and may explain why we did not observe an increase in expression level of this gene 24 h after handling stress. *Hsp70* responses in relation to other stressors (e.g., temperature) seem to be in agreement with this explanation. Petkeviciute et al. (2015) found a 63.8-fold increase in *hsp70* transcripts for *A. tonsa* after 45 min exposure to 30°C. This corresponds with the findings of Rahlff et al. (2017), where a heat shock of 28°C for 3 h resulted in significant up-regulation of *hsp70*, which was measurable after 30 min and peaked with a 185-fold increase after 1.5 h. A smaller peak of 60.4-fold increase remained 4 h after the heat shock (Rahlff et al., 2017). The peak in *hsp70* expression that occurred a few hours after exposure, which subsequently declined, could explain our findings. In general, the expression levels observed here were low, implying that *hsp70* and a number of other genes may show peak up-regulation within 24 h after exposure to stressors.

The down-regulated *hsp70* 24 h post exposure to salinity was enriched for the nucleus (CC, **Table 6**), precatalytic spliceosome (CC, **Table 6**), presynapse (Supplementary Material S5), late endosomal microautophagy (BP, Supplementary Material S5), cellular response to topologically incorrect protein (BP, Supplementary Material S5), and perichromatin fibrils CC, Supplementary Material S5). Many of these terms are stress-related, and could have included additional GO-terms that are relevant in relation to *hsp70* "chaperone mediated protein folding" and "*de novo* protein folding" (Supplementary Material S5).

It is noteworthy that aldehyde-dehydrogenases (*ALDH2* and *ALDH7A1*), ubiquitin, and related genes (e.g., *UBP25*, *FANCL*, *UBR2, UBR4*, *UBE2C*, **Table 5**, Supplementary Material S1; https://figshare.com/articles/S1a_Trinotate_Annotation_A_tonsa_xls/5928799/1, Supplementary Material Table 5), which have been used in copepods as transcriptional biomarkers, are among the overlapping DEGs 24 h post exposure to handling stress or salinity shock (e.g., Lauritano et al., 2011, 2016). Biomarkers should, thus, be carefully selected to avoid artifacts caused by handling stress in the analysis of gene expression.

In summary, handling stress clearly affects both biomarkers and transcriptome-wide patterns of differential gene expression of *A. tonsa,* and these stress responses probably take place within 24 h after exposure to a stressor. Some of the differentially expressed genes were in common between the handling stress and salinity shock treatments, suggesting that these may play an important role in protection against multiple stressors. Due to the small, but significant differences in expression levels of some of the commonly used biomarkers, these genes should be used with caution in stress-related studies, since they potentially peak within 24 h after exposure.

The limited response at the transcriptional level 15 min following exposure to handling stress suggests that organisms collected in plankton tows of short duration and immediately used in incubation experiments or being preserved are likely to exhibit transcriptional profiles that represent their *in situ* physiological state (e.g., Häfker et al., 2017). It is, however, important to be aware of that handling has the potential to affect gene expression regardless of animal species. Thus, handling should therefore be considered as a factor when examining stress at the transcriptional level.

DATA AVAILABILITY

RNA-Seq data available in the NCBI Sequence Read Archive with the bio-project accession number PRJNA407266.

AUTHOR CONTRIBUTIONS

BN, PJ, AB, and BH conceived the study; BN, PJ, AB, and BH designed the experiments; BN, PJ, and AB collected and analyzed the data; BN wrote the paper; BN, PJ, AB, and BH contributed substantially to interpreting the data and developing the manuscript, and take full responsibility for the content of the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00156/full#supplementary-material

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