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Identification of molecular markers associated with starvation in female *Calanus sinicus* (Copepoda: Calanoida)

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ABSTRACT: Evaluation of the physiological states of copepods can improve current understanding of the dynamics of zooplankton population and contribute to assessments of the health of trophic linkages in marine ecosystems. However, assessing the starvation status of copepods *in situ* using conventional methods is difficult because of spatial and temporal changes in food availability. Here, we analyzed gene expression to identify the molecular markers associated with starvation in the copepod *Calanus sinicus* from the western North Pacific Ocean. Differentially expressed genes (DEGs) were compared between starved and satiated copepods using high-throughput mRNA sequencing (RNA-Seq). Sixteen DEGs were identified. Nine of these DEGs were assigned to known functional genes associated with energy metabolism, egg production, and somatic growth, and therefore were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR) as candidate markers for starvation. To evaluate the reliability of these candidate markers as indicators for starvation, copepods were given different food concentrations and fasted for various periods. Gene expression was highly sensitive to temporal and quantitative changes in food conditions. During starvation, the expression of NADH-dehydrogenase (related to energy metabolism) was upregulated and that of vitellogenin 2 (related to egg production) was downregulated compared to their expression in satiated copepods. As might be expected from such changes in expression, complete fasting significantly decreased egg production. Changes in NADH-dehydrogenase and vitellogenin 2 expression were therefore correlated with the degree of copepod starvation or food limitation. This information may help to assess potential reproductivity and predict egg production rates in copepods in their natural environment.

KEY WORDS: Copepod · Starvation · Molecular marker · Gene expression · RNA-Seq · qRT-PCR

1. INTRODUCTION

Marine plankton rapidly respond to environmental changes. Evaluating the physiological state of plankton is crucial for understanding how these organisms respond under varying environmental conditions and for assessing the effects of environmental variability on the structure and function of planktonic marine ecosystems (Hays et al. 2005, Allen & Polimene 2011). Food availability to planktonic organisms varies spatially (over a wide range of scales from several kilometers to few centimeters; Mullin & Brooks 1976, Dagg 1977, Levasseur et al. 1983) and temporally (e.g. on seasonal, diurnal, and hourly scales; Platt 1975, Dagg 1977, Hassett & Landry 1988). Marine planktonic environments tend to be nutritionally dilute, with food resources dispersed at low concentrations (Kiørboe 2011). Therefore, food limitation in the natural environment can occur in various areas and seasons and may affect the growth and reproduction of zooplankton (Threlkeld 1976, Hassett & Landry 1990). In contrast, McLaren (1978) and Cor-

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kett & McLaren (1979) noted that copepod development rates in the natural environment are unaffected by fluctuations in food availability. Copepods are the most abundant and well-studied zooplankton taxa in marine ecosystems (Humes 1994, Brun et al. 2017). Under experimental conditions, various studies have reported negative effects of starvation on copepod productivity. Fasting reduces copepod metabolic rates; they conserve material and energy, and decrease their growth, development, and reproduction rates (Mayzaud 1976, Dagg 1977, Landry & Hassett 1985, Yebra et al. 2011). The physiological states of copepods have been evaluated based on metabolic parameters (e.g. respiration rate, egg production rate, enzyme activity, and RNA/DNA ratio; Hassett & Landry 1990, Durbin & Durbin 1992, Niehoff 2000, Thor 2003, Kreibich et al. 2008, Ning et al. 2013, Kobari et al. 2017) and indirect estimates from environmental parameters (water temperature and food distribution, composition, quality, and quantity; Runge 1980, Uriarte & Villate 2006, Saiz & Calbet 2007). In the natural environment, however, the physiological state of copepods is not easily determined. Conventional analytical methods are not easily applied to copepods in environments with heterogeneous food availability. Measurements of egg production and respiration rate require laborious incubations, which limits their application to the analysis of a small number of samples. Moreover, these incubation methods cannot completely exclude the influences of manipulation and of the artificial conditions. The use of biochemical approaches that focus on protein synthesis activity, such as the RNA/DNA ratio and the aminoacyl tRNA synthetase (AARS) activity analysis, overcomes these drawbacks by eliminating the need for incubations. However, although protein synthesis activity is a good indicator of metabolic rate, the conventional biochemical approaches cannot determine the types of protein an animal does or does not need; therefore, analyzing protein synthesis activity of an individual is insufficient to reveal physiological changes. Even though copepods with low metabolic rates, characterized by declines in egg production rate, respiration rate, or protein synthesis activity, have been observed in their natural environment, whether these declines are derived from starvation remains unclear. Therefore, a sensitive and direct approach that evaluates the physiological state of starved copepods in their natural environment is needed.

Physiological states can be directly measured by investigating the expression of specific target genes. High-throughput mRNA sequencing (RNA-Seq) can be applied to copepods for which genome information is limited (Haas et al. 2013, Conesa et al. 2016). This methodology has been used to identify the physiological responses of copepods to environmental stresses wuch as high temperature (Lima & Willett 2017), acidification (Bailey et al. 2017), toxic algae (Roncalli et al. 2016), and heavy metal pollution (Wang et al. 2017). Changes in gene expression precede physiological responses such as changes in respiration rate and egg production rate, which can be measured using conventional methods. Moreover, the function of a gene and its expression suggest what type of protein an animal does or does not need. Therefore, the characterization of gene expression should provide sensitive and meaningful insights into the physiological responses of an organism to various environmental changes. Candidate molecular markers associated with starvation and their sequences can be identified using RNA-Seq. The expression of these markers can then be determined quickly and easily by quantitative real-time PCR (qRT-PCR), and target gene quantification can be used on field-collected zooplankton to identify starved copepods in their natural environment without incubations.

The present study aimed to identify molecular markers associated with starvation in female *Calanus sinicus*. This abundant calanoid copepod is indigenous to the continental shelf of the subtropical western North Pacific Ocean. *Calanus sinicus* is an ecologically important zooplankton species because it plays a key role in marine food webs by linking primary producers to higher trophic levels (Uye 2000, Pu et al. 2004a). We identified differentially expressed genes (DEGs) in starved females by RNA-Seq, and DEGs whose functions could be estimated from the results of homology search were selected as candidate markers. We then used qRT-PCR to identify changes in expression under various food availabilities and to validate candidate genes as markers for starved copepods.

2. MATERIALS AND METHODS

2.1. Identification of candidate molecular markers for starvation by RNA-Seq

2.1.1. Sample collection and starvation experiment for RNA-Seq analysis

A starvation experiment was conducted on board the RV 'Soyo-Maru' during an O-line transect cruise across the Kuroshio Current 138° E off the southern

coast of Japan in October 2015. Zooplankton were collected with a plankton net (80 cm mouth opening, 100 µm mesh size, 3 l cod-end) at the sampling station in the continental slope water area shoreward of the Kuroshio Current (34° 00′ N, 138° 00′ E). The net was towed vertically at 0.1 m s^{-1} and 0–100 m depth. Specimens were immediately transferred to filtered surface seawater in a cooling container. Adult female *Calanus sinicus* were sorted from the bulk sample with a large bore pipette under a stereomicroscope. Surface seawater for bottle incubation was collected using the ship's built-in pump. For the starvation treatment (STV), 12 females were transferred to a 3-l bottle filled with 0.8-µm-filtered surface seawater. For the fed treatment (FED), 12 females were transferred to a 3-l bottle filled with feed-contained seawater; feed particles (size >20 µm) were collected from the surface seawater using a 20-µm net and the provided particle concentration was equivalent to that in the field. The bottles were maintained in the dark at 18°C. At this temperature, complete fasting can affect the physiological condition of *C. sinicus* without causing high mortality, depending on the fasting period (Pu et al. 2004b). After 24 h, approximately twice as long as the food limitation associated with diel vertical migration in natural environments (Runge 1980), each of the 12 individuals from each group was transferred to 1.5 ml RNAlater (Ambion) and stored at −80°C until RNA extraction.

2.1.2. RNA extraction, cDNA library preparation, and Illumina sequencing

Triplicate samples were prepared by pooling female *C. sinicus* from each FED and STV group. Four individuals were pooled in each replicate. Total RNA was extracted with TRIzol Reagent (Invitrogen) and purified with the RNeasy Mini Kit (Qiagen). Genomic DNA was eliminated by treatment with DNase I (Sigma-Aldrich). Total RNA was measured with a Qubit 2.0 Fluorometer (Thermo Fisher Scientific), and its quality was confirmed with the RNA 6000 Pico LabChip on an Agilent 2100 Bioanalyzer. Approximately 500 ng of high-quality RNA was used for cDNA library preparation. Library preparation was performed with the TruSeq RNA sample preparation kit v. 2 (Illumina). Briefly, mRNA was isolated from total RNA with magnetic oligo dT beads, which were then fragmented and used in first- and second-strand cDNA synthesis. The cDNA fragments were ligated with index adapters to discriminate samples and amplified by PCR to enrich fragments with adapters (cDNA library). For the final 15-µl cDNA libraries, an average ~260 bp length was confirmed using the Agilent 2100 Bioanalyzer and a DNA 1000 LabChip. Library concentrations of 58.5–94.4 ng $µl^{-1}$ were measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Equal quantities of each cDNA library were pooled and sequenced by the Beijing Genomics Institute (BGI) on an Illumina HiSeq 4000 in the 100-bp paired-end mode.

2.1.3. De novo assembly and differential gene expression analysis by RNA-Seq

Raw reads were cleaned by filtering out adapter-bearing and low-quality reads with FASTX-Toolkit v. 0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit/). Low-quality reads were defined as those with >40% of bases with a Phred score of <20. The clean reads were assembled into contiguous sequences (contigs) using Trinity v. 2.0.6 (Grabherr et al. 2011) with a minimum contig length of 500 bp. TransDecoder v. 3.0.0 (Torrens-Spence et al. 2016) was used to extract coding sequences (CDSs) from the contigs as transcripts (minimum length 300 bp). Redundant transcript sequences were removed with Cd-hit v. 4.6.6 (Li & Godzik 2006) at a cut-off of 100% sequence similarity of the transcript. Transcriptome assembly and annotation completeness were assessed by benchmarking universal single-copy orthologs (BUSCO) v. 3.0.1 analysis (Simão et al. 2015) based on a set of 303 single-copy conserved eukaryotic genes (downloaded October 2018), and basic local alignment search tool (BLAST)x v. 2.3.0 (Altschul et al. 1997) analysis against the National Center for Biotechnology Information (NCBI) non-redundant (nr) protein database (updated July 2018) with an E-value $<1 \times 10^{-5}$. All reads and assembled transcripts were deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (SRA) and Transcriptome Shotgun Assembly (TSA) division under the DDBJ BioProject (accession number PRJDB7229), respectively. The quality-filtered reads in each sample were mapped back to the reference transcript sequences with Bowtie2 v. 2.2.9 (Langmead & Salzberg 2012). The software package RNA-Seq by Expectation Maximization (RSEM) v. 1.2.31 (Li & Dewey 2011) was used to calculate the number of mapping reads per transcript and to quantify gene expression based on a maximum likelihood estimate. The DEGs were identified with DESeq2 v. 1.12.4 in R (Love et al. 2014). A DEG was defined as significant when $|\log_2$ fold change ($\log_2$FC)$| \geq 1$ and the adjusted p-value (false discovery rate, FDR) was

<0.01. Gene descriptions were obtained by using the BLAST Description Annotator tool in Blast2GO v. 5.1.13 (Conesa et al. 2005) based on the top 10 BLAST hits. The DEG was categorized based on the function of its encoded protein.

2.2. Validation of candidate markers by qRT-PCR

2.2.1. Experimental design for qRT-PCR analysis and egg production rate measurement

Zooplankton samples were collected from the RV 'Tachibana' from Yokohama National University in western Sagami Bay in southern Japan (35° 09′ N, 139° 10′ E) during 4 different months (Table 1). The sampling technique used for the RNA-Seq analysis was also used here. Adult female *C. sinicus* were sorted in the laboratory and individually transferred to 60-ml bottles fitted with 200-µm mesh screens at the bottom to prevent egg cannibalism. Each bottle held one adult female. *Tetraselmis* sp. was added as a food source to all bottles at a density of 8000 cells ml^{-1}. The copepods were acclimated to surplus food for 48 h before starvation. The bottles were maintained in an incubator at 18°C and a 14 h light:10 h dark cycle. The acclimated copepods were then divided into 2 groups, each transferred into a 60-ml bottle filled either with GF/F (Whatman) filtered surface seawater (STV treatment) or 8000 cells ml^{-1} *Tetraselmis* sp. (FED treatment). Each bottle held one adult female. For comparing the response to starvation in terms of gene expression, we incubated copepods under different fasting periods (3, 6, 12, 24, and 72 h). After each experimental period, copepods from each bottle (Table 1) were preserved in RNAlater (1.5 ml) and frozen (–80°C) until RNA extraction. Female egg production rate (EPR; eggs $copepod^{-1}$ d^{-1}), a conventional metabolic parameter, was measured to ensure that females in the FED treatment were healthy and satiated during the experiments. A decline in EPR associated with food limitation in the STV treatment would indicate that changes in the copepods' physiological condition have appeared. Eggs were counted every 24 h to determine EPR during the experiment. *Calanus sinicus* spawning has a diel periodicity (Huang et al. 1992). Therefore, at least 24 h incubation is required to determine EPR (Hirche et al. 1997), which was calculated for each individual at each counting time (Table 2). Because some individuals were removed at each experimental period and preserved for RNA extraction, the number of individuals used for EPR measurement was lower than the initial number of individuals. In addition, 5 different food concentrations (0, 500, 1000, 2000, and 8000 cells ml^{-1}) were provided and tested after 48 h incubation to identify any correlation between gene expression, EPR, and food concentration. Chlorophyll *a* levels at these food concentrations were determined from fluorescence measurements (Holm-Hansen et al. 1965) using a Turner Designs fluorometer.

2.2.2. RNA extraction, cDNA synthesis, and qRT-PCR analysis

Total RNA extraction and measurement of its concentration and quality were conducted for each individual as performed for the RNA-Seq analysis. Approximately 500 ng of each total RNA sample was reverse-transcribed into first-strand cDNA using PrimeScript RT master mix (TaKaRa Bio) following the manufacturer's instructions and the GeneAmp PCR System 9700 (Applied Biosystems). Oligonucleotide

Table 1. Sampling month and number of *Calanus sinicus* female adults used in each experiment for qRT-PCR analyses. FED: fed treatment; STV: starvation treatment; –: no sample

Starvation experiments

Date	0 h	3 h		6 h		12 h		24 h		72 h	
	FED	FED	STV	FED	STV	FED	STV	FED	STV	FED	STV
January 2017	3	–	–	–	–	3	3	3	3	3	1
October 2017	3	–	4	–	3	–	3	–	–	–	–
December 2017	6	5	5	5	5	5	5	5	5	5	6
Total	12	5	9	5	8	8	11	8	8	8	7

Different food concentration experiment

Date	Food concentration (cells ml^{-1})				
	8000	2000	1000	500	0
November 2017	3	4	4	4	4

Table 2. Sampling month and number of *Calanus sinicus* female adults used in each experiment for egg production rate (EPR). FED: fed treatment; STV: starvation treatment; –: no sample

Starvation experiments										
Date	−24 h		0 h		24 h		48 h		72 h	
	FED	STV	FED	STV	FED	STV	FED	STV	FED	STV
January 2017	20	12	16	12	8	8	4	4	3	1
October 2017	13	–	13	–	–	–	–	–	–	–
November 2017	15	4	15	4	3	4	3	4	–	–
December 2017	31	26	31	26	10	16	5	6	5	6

Different food concentration experiment					
Date	Food concentration (cells ml^{-1})				
	8000	2000	1000	500	0
November 2017	3	4	4	4	4

primers to amplify specific fragments of candidate markers and hypothetical reference genes were designed with Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and their sequences are shown in Table 3. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Actin, and 16S rRNA — commonly used as reference genes in qRT-PCR analyses — were selected as hypothetical reference genes. Primers for the 16S rRNA gene were designed from a *C. sinicus* sequence available in GenBank (accession number KT960998). As GAPDH and Actin sequences for *C. sinicus* are not available in GenBank, primers for these genes were designed from homologous *C. finmarchicus* sequences (GAPDH: accession number ES387158; Actin: accession number ES387224). BestKeeper (Pfaffl et al. 2004) and NormFinder (Andersen et al. 2004) were used to identify the best reference gene for our starvation experiment. BestKeeper determines gene expression variation based on cycle threshold (C_t) values, calculating standard deviation (SD [$\pm C_t$]) and coefficient of variance (CV [$\%C_t$]). Genes with SD < 1 and CV < 2 are considered to be stable (Pfaffl et al. 2004). BestKeeper revealed that the 16S rRNA gene (SD = 0.15, CV = 1.53) was stable. In contrast, the GAPDH (SD = 0.85, CV = 4.13) and Actin (SD = 1.12, CV = 5.20) genes showed greater variation than the 16S rRNA gene, indicating that neither of them was suitable as a reference gene in this study. NormFinder ranks average expression stability values; the lowest value has the greatest stability. The NormFinder analysis indicated that the 16S rRNA gene (stability value = 0.03) was the most stable of the 3, followed by the GAPDH (stability value = 0.05) and the Actin (stability value = 0.07) genes. As both analyses identified the 16S rRNA gene as the most stably expressed, it was used as reference in this study.

Table 3. Genes and primer sequences used in this study

Gene name	Primer sequences
ATP-dependent RNA helicase	F:5′-TGGAGCCCTTACACAGCTTGG-3′ R:5′-AAGAAGCATTGTCCTGCTGGC-3′
Small ubiquitin-related modifier	F:5′-TTCACGCCAGGATCGAGTACA-3′ R:5′-TAAGCCTTCCTCAGCTTCCCC-3′
Xylosyltransferase	F:5′-ATTCGCGGGTGTGCAGTATTC-3′ R:5′-CTGCGTGCTATCCCCAGAACA-3′
NADH-dehydrogenase	F:5′-TGCTACAGACTGGCTGCAAA-3′ R:5′-TAGAGTAGTCATGGCCCCCA-3′
Cytochrome *c* oxidase	F:5′-AGATGGATCCTCCTCCATATGTTCC-3′ R:5′-GTGTGGCTGTTGTGGATCAGAG-3′
Short-chain dehydrogenase	F:5′-GATGCTGGCTCAGTCACTTGC-3′ R:5′-TGTGCAATCTGGTCAGGGTCC-3′
Vitellogenin 2	F:5′-ACCAATGTAGCCAGAGGCAC-3′ R:5′-CCCACGAAGGGAAGCAATGA-3′
Singed wings 2	F:5′-CAGTTGCATGACCAGGGGTTG-3′ R:5′-CTTCTTTGCTGGCAACCCCTG-3′
Vitellogenin 1	F:5′-TCTGCTGAAATGCAGGCTGTG-3′ R:5′-GAGCGGAAGCCTGTGTTCTTG-3′
16S rRNA	F:5′-AGAAGATCCTCTAGGGATAACAGCA-3′ R:5′-AACAGACCGTCTCCAGTGAGC-3′
Actin	F:5′-AAGCCAAGGCACTTGACTCCA-3′ R:5′-AGCATGCTCCAATGCAAGACC-3′
GAPDH	F:5′-TCAATGAGCGTGACCCTGCTA-3′ R:5′-CAGCAGAAGGAGCGGAGATGAT-3′

qRT-PCR was performed in the LightCycler 480 II Real-Time PCR System (Roche Life Science) with a SYBR Premix Ex Taq Kit (TaKaRa Bio). The total reaction volume was 10 µl and consisted of 5 µl SYBR Premix, 0.2 µl of each primer, 1 µl cDNA, and 3.6 µl RNase-free dH_2O. The qRT-PCR profile was as follows: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. The initial cDNA sample, taken immediately after surplus food acclimation, was used as the control to calculate the relative expression of each candidate gene in each treatment. Relative gene expression was measured from standard curves based on C_t and was normalized to the reference gene encoding 16S rRNA by the efficiency-corrected $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001).

2.2.3. Statistical analyses

Normality and homogeneity of variances were assessed by the Kolmogorov-Smirnov test and the *F*-test or Levene's test, respectively. Differences in gene expression between the FED and STV groups for each fasting period were assessed with Welch's *t*-tests. Differences in gene expression in each treatment among culturing times and food concentrations were investigated with one-way ANOVA followed by Tukey's multiple comparisons test. The difference in EPR between the FED and STV groups for each fasting period was assessed with a Mann–Whitney *U*-test. Differences in EPR in each treatment among culturing times and food concentrations were also investigated by ANOVA followed by Tukey's multiple comparisons test. In all cases, differences were considered significant at $p < 0.05$. The statistical analyses were performed in R v. 3.3.1 (R Core Team 2016) using RStudio v. 1.1.419 (https://www.rstudio.com).

3. RESULTS

3.1. Identification of candidate molecular markers for starvation by RNA-Seq analysis

The Illumina HiSeq 4000 sequencing yielded 480 608 454 raw reads. Filtering out the adapter-bearing and low-quality reads resulted in 371 095 980 clean reads. *De novo* assembly of the clean reads generated 107 959 contigs with 500–17 081 bp. The contiguity index N_{50}, a weighted median statistic such that 50% of the entire assembly is contained in contigs equal to or larger than this value, was 1273 bp. After extracting the CDSs and removing the duplicate sequences, 84 095 non-redundant reference transcript sequences were retained, ranging from 300 to 16 680 bp in length and with N_{50} = 1104 bp. The BUSCO analysis reported 281 complete (92.8%), 14 fragmented (4.6%), and 8 missing (2.6%) eukaryotic single-copy genes among the 303 examined. The BLASTx analysis revealed homology with sequences deposited in the nr database for 53 387 of the 84 095 sequences (63.5%), and 33 528 of the homologous sequences (62.8%) were derived from *Eurytemora affinis*, followed by 518 sequences (0.6%) derived from *Cryptotermes secundus*. The clean reads in each sample were mapped back to the transcript sequences. An overall alignment rate of 55.1–57.8% was obtained, and 42.0–44.9% of the clean reads aligned concordantly (paired reads aligned with the appropriate orientation and distance) exactly once, while 13.2–14.2% of the clean reads aligned concordantly more than once.

Sixteen sequences were significantly identified as DEGs (Fig. 1). Of these 16 DEGs, 11 were upregulated and 5 were downregulated for STV. The BLASTx analysis indicated that 6 and 3 genes were annotated as genes with upregulation and downregulation in the STV group, respectively (Table 4). No homologous sequences were found for the other 7 DEGs.

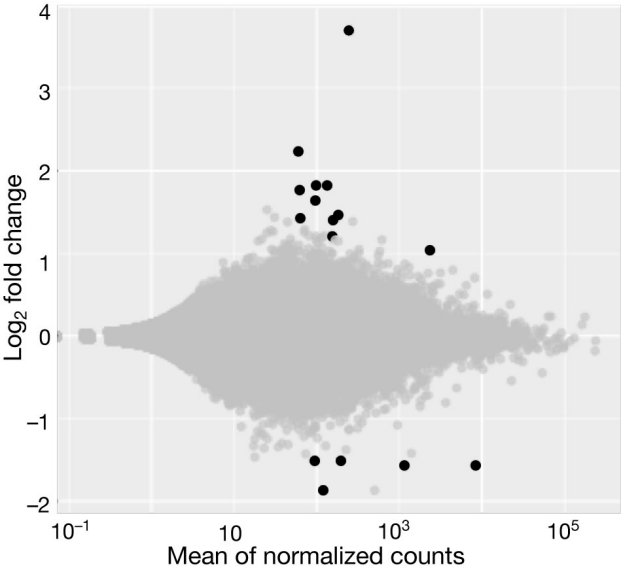

Fig. 1. Representative MA plot (where M = log ratio and A = average) identifying genes with significant differential expression ($|\log_2$ fold change$| \geq 1$ and false discovery rate < 0.01) between the fed (FED) and starvation (STV) treatments. The *x*-axis represents the mean of the normalized counts. The *y*-axis represents the change in expression of each gene on a $\log_2$ fold-change basis (STV relative to FED). Each black dot represents a differentially expressed gene (DEG). Each gray dot indicates a non-DEG

The DEGs with BLAST hits were named according to their similarity to homologous sequences in the NCBI nr protein database. Each DEG was categorized based on the function of its encoded protein. We categorized DEGs as energy metabolism (*ND*: NADH-dehydrogenase; *COX*: cytochrome *c* oxidase; *SD*: short-chain dehydrogenase), glycosaminoglycan biosynthesis (*XYLT*: xylosyltransferase), protein function and synthesis activity modification (*DHX*: ATP-dependent RNA helicase; *SUMO*: small ubiquitin-related modifier), egg production (*VTG1*: vitellogenin 1; *VTG2*: vitellogenin 2), and somatic growth (*SW2*: singed wings 2) genes. NADH-dehydrogenase catalyzes the transfer of electrons from NADH to coenzyme Q10, and in eukaryotes it is located in the inner membrane of the mitochondria (Weiss et al. 1991); it is the first enzyme of the oxidative phosphorylation (OXPHOS) system within the mitochondrial inner membrane. The OXPHOS system refers to a series of phosphorylation (ATP synthesis) reactions that occur in conjugation with the electron transport system. Cytochrome *c* oxidase catalyzes the final step of the mitochondrial electron transfer chain and is one of the major regulators for the OXPHOS system (Capaldi 1990). Short-chain dehydrogenase is a very large family of enzymes, most of which are known to be NAD- or NADP-dependent oxidoreductases (Jörnvall et al. 1995). NAD(P)-dependent oxidoreductases catalyze the transfer of electrons from an electron donor to an electron acceptor. Part of this enzyme group forms electron transfer chains in mitochondria. Xylosyltransferases catalyze the first sequential glycosyltransferase reaction required for synthesis of the chondroitin sulfate-protein linkage region and formation of the polysaccharide chain (Stoolmiller et al. 1972). ATP-dependent RNA helicase unwinds RNA duplexes and plays important roles in many fundamental processes in the cell, including mRNA splicing, ribosome assembly, and translation (Fuller-Pace 1994). Small ubiquitin-related modifier is a protein that temporarily binds to other proteins in the cell and helps its stabilization, transport, or transcription regulation (Bayer et al. 1998). Vitellogenin is a precursor protein of egg yolk, which is utilized as a source of nutrients by developing embryos of oviparous organisms (Wahli 1988). Finally, the singed wings protein plays a role in the

Table 4. List of differentially expressed genes (DEGs) between fed and starvation treatments in adult female *Calanus sinicus* transcriptomes obtained by RNA-Seq. Length: length of transcript reconstructed in this study (bp); $\log_2$FC: expression in the starvation treatment in relation to that in the fed treatment; FDR: false discovery rate; E-value: top-hit BLAST result E-value; similarity: average similarity of the top 10 BLAST hits; N/A: not applicable

Trinity transcript	Length (bp)	$\log_2$FC	FDR	Description	Abbreviation	E-value	Similarity (%)	Top hit species	Accession no.
Upregulation									
comp952482_c0	3993	3.7	5.91×10^{-27}	ATP-dependent RNA helicase	*DHX*	5.78×10^{-75}	56.8	*Athalia rosae*	XP_012250687
comp934074_c0	501	2.2	1.32×10^{-6}	Small ubiquitin-related modifier	*SUMO*	6.88×10^{-28}	73.6	*Caenorhabditis nigoni*	PIC13693
comp934236_c0	1224	1.8	3.97×10^{-4}	N/A					
comp940563_c0	4974	1.8	4.06×10^{-4}	N/A					
comp930645_c0	3345	1.8	8.22×10^{-4}	Xylosyltransferase	*XYLT*	1.85×10^{-16}	65.1	*Ceratitis capitata*	XP_004518981
comp940188_c1	339	1.0	8.22×10^{-4}	NADH-dehydrogenase	*ND*	1.14×10^{-42}	62.4	*Tortanus dextrilobatus*	ALS05198
comp937948_c0	4251	1.6	3.18×10^{-3}	N/A					
comp936344_c1	360	1.5	3.51×10^{-3}	Cytochrome *c* oxidase	*COX*	1.02×10^{-19}	64.6	*Astyanax mexicanus*	XP_007247191
comp937934_c0	639	1.4	3.67×10^{-3}	N/A					
comp951423_c0	720	1.2	4.01×10^{-3}	Short-chain dehydrogenase	*SD*	3.76×10^{-53}	59.9	*Eurytemora affinis*	XP_023345642
comp956256_c0	975	1.4	4.01×10^{-3}	N/A					
Downregulation									
comp916707_c0	1869	–1.9	2.79×10^{-4}	N/A					
comp958909_c0	1005	–1.6	2.79×10^{-4}	Vitellogenin 2	*VTG2*	8.77×10^{-30}	53.2	*Eurytemora affinis*	XP_023333850
comp958549_c0	6108	–1.5	3.97×10^{-4}	Singed wings 2	*SW2*	3.74×10^{-21}	50.5	*Zootermopsis nevadensis*	XP_021914722
comp958909_c1	4362	–1.6	4.06×10^{-4}	Vitellogenin 1	*VTG1*	3.84×10^{-152}	51.3	*Eurytemora affinis*	XP_023332353
comp956579_c0	1071	–1.5	6.91×10^{-3}	N/A					

ecdysone-induced cascade (Schwartz et al. 2004); it is thought to be related to the indirect control of ecdysone genes. The 9 aforementioned DEGs were selected as candidate molecular markers for starvation.

3.2. Validation of candidate markers by qRT-PCR

3.2.1. Gene expression patterns for different fasting periods

The qRT-PCR performed on the 9 candidate molecular markers using specimens from the time-series starvation experiment (0, 3, 6, 12, 24, and 72 h; Fig. 2) revealed that the expression of *ND* and *VTG2* was consistently regulated with fasting time in the STV group. The expression of both genes in the FED group was relatively stable compared to the changes in the expression of these genes in the STV group. In the FED group, the expression of *ND* remained low during the experiment, and no significant differences were detected (ANOVA: $F_{5,25}$ = 1.617, p = 0.192). In contrast, the expression of *ND* in the STV group increased with fasting time. Significant differences in expression among fasting times were found for the STV group (ANOVA: $F_{5,26}$ = 9.733, p = 0.000). Tukey's test showed significant differences in expression between 24–72 and 0–3 h (p = 0.000–0.013). After 6 h, the differences in expression between the FED and STV groups remained significant (*t*-test: p = 0.000–0.011).

In the FED group, the expression of *VTG2* was stable during the experiment, and no significant differences were detected (ANOVA: $F_{5,25}$ = 0.571, p = 0.721). In contrast, the expression of *VTG2* in the STV group declined at 6 h and remained very low thereafter. Significant differences in expression among fasting times were found in the STV group ($F_{5,26}$ = 5.998, p = 0.001). Tukey's test showed significant differences in expression between 24–72 h and 0–3 h (p = 0.006–0.013). After 6 h, the expression of *VTG2* remained significantly lower in the STV group than in the FED group (*t*-test: p = 0.000–0.047).

After 24 h, the expression of *COX* and *VTG1* was significantly different between the 2 groups. The difference in expression of *COX* between the FED and STV groups was significant at 24 and 72 h (*t*-test: p = 0.018 and 0.003, respectively). However, the expression of *COX* was not stable in the FED group, and there was a significant difference in expression between 0 and 72 h (ANOVA: $F_{5,25}$ = 3.393, p = 0.018; Tukey's test: p = 0.013). Differences in expression of *VTG1* between the FED and STV groups were significant at 24 and 72h (*t*-test: p = 0.000 and 0.002, respectively). Although no significant difference was found in the FED group (ANOVA: $F_{5,25}$ = 1.028, p = 0.423), the variation in expression of *VTG1* was relatively greater than that of *VTG2*. A temporary significant difference in the expression of *SD* was found between the FED and STV groups at 12 h (*t*-test: p = 0.044). There were no significant differences between the FED and STV groups in *XYLT*, *SW2*, *DHX*, or *SUMO* expression at any fasting period. None of these 4 genes was consistently upregulated or downregulated with time. In certain individuals, the expression of *SUMO* and *DHX* was below the detection limit.

3.2.2. Gene expression patterns with different food concentrations

The expression of *ND* was upregulated and that of *VTG1* and *VTG2* was downregulated with decreasing food concentration (Fig. 3). There were significant differences in the expression of *ND* in the 0 and 500 cells ml^{-1} treatment groups compared with that in the 8000 cells ml^{-1} treatment group (ANOVA: $F_{4,14}$ = 3.393, p = 0.038; Tukey's test: p = 0.045 and 0.048, respectively). There were significant differences in the expression of *VTG2* in the 0 cells ml^{-1} treatment group compared with that in the 2000 and 8000 cells ml^{-1} treatment group (ANOVA: $F_{4,14}$ = 7.601, p = 0.002; Tukey's test: p = 0.003 and 0.03, respectively). There were significant differences in the expression of *VTG1* in the 0, 500, and 1000 cells ml^{-1} treatment groups compared with that in the 8000 cells ml^{-1} treatment group (ANOVA: $F_{4,14}$ = 6.256, p = 0.004; Tukey's test: p = 0.007, 0.045, and 0.016, respectively). There were no clear correlations between food concentration and the expression of *COX*, *SD*, *XYLT*, *SW2*, *DHX*, or *SUMO.* None of the differences were significant according to ANOVA ($F_{4,14}$ = 0.301–2.203, p = 0.122–0.873).

3.2.3. EPR

The EPR in the FED group was relatively constant in each month (Fig. 4). According to ANOVA, no significant differences in EPR were detected for the FED group in any month except in November. In November, significant differences were observed at 0 h compared to –24 and 24 h (ANOVA: $F_{3,44}$ = 5.146, p = 0.004; Tukey's test: p = 0.009 and 0.006, respectively).

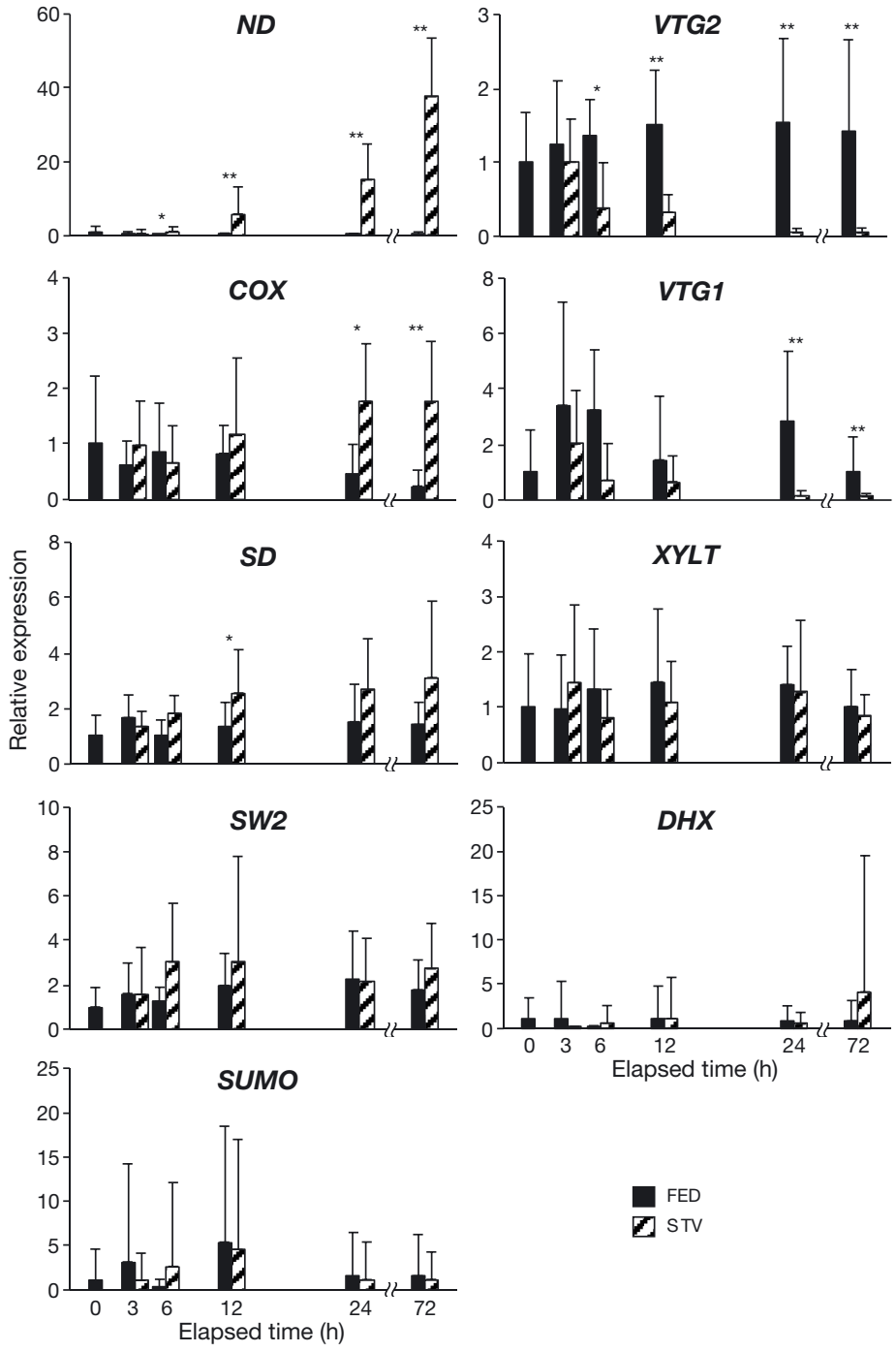

Fig. 2. Average relative expression of candidate markers in the fed (FED) and starvation (STV) treatments. Expression was measured by qRT-PCR and compared against values measured in individuals at the start of the experiment (elapsed time = 0 h). *ND*: NADH-dehydrogenase; *VTG2*: vitellogenin 2; *COX*: cytochrome *c* oxidase; *SD*: short-chain dehydrogenase; *VTG1*: vitellogenin 1; *XYLT*: xylosyltransferase; *SW2*: singed wings 2; *DHX*: ATP-dependent RNA helicase; *SUMO*: small ubiquitin-related modifier. The error bars show ±SD. *$p < 0.05$; **$p < 0.01$ (Welch's *t*-test between FED and STV at each time point)

In contrast, the EPR in the STV group clearly decreased after 48 h and fell to 0 after 72 h in all months, but EPR did not decrease for up to 24 h after fasting. There were significant monthly differences in EPR between 0 and >48 h in the STV group (in January, ANOVA: $F_{4,53} = 4.684$, $p = 0.003$; Tukey's test: $p = 0.031–0.038$; in November, ANOVA: $F_{3,16} = 5.354$, $p = 0.010$; Tukey's test: $p = 0.010$; in December,

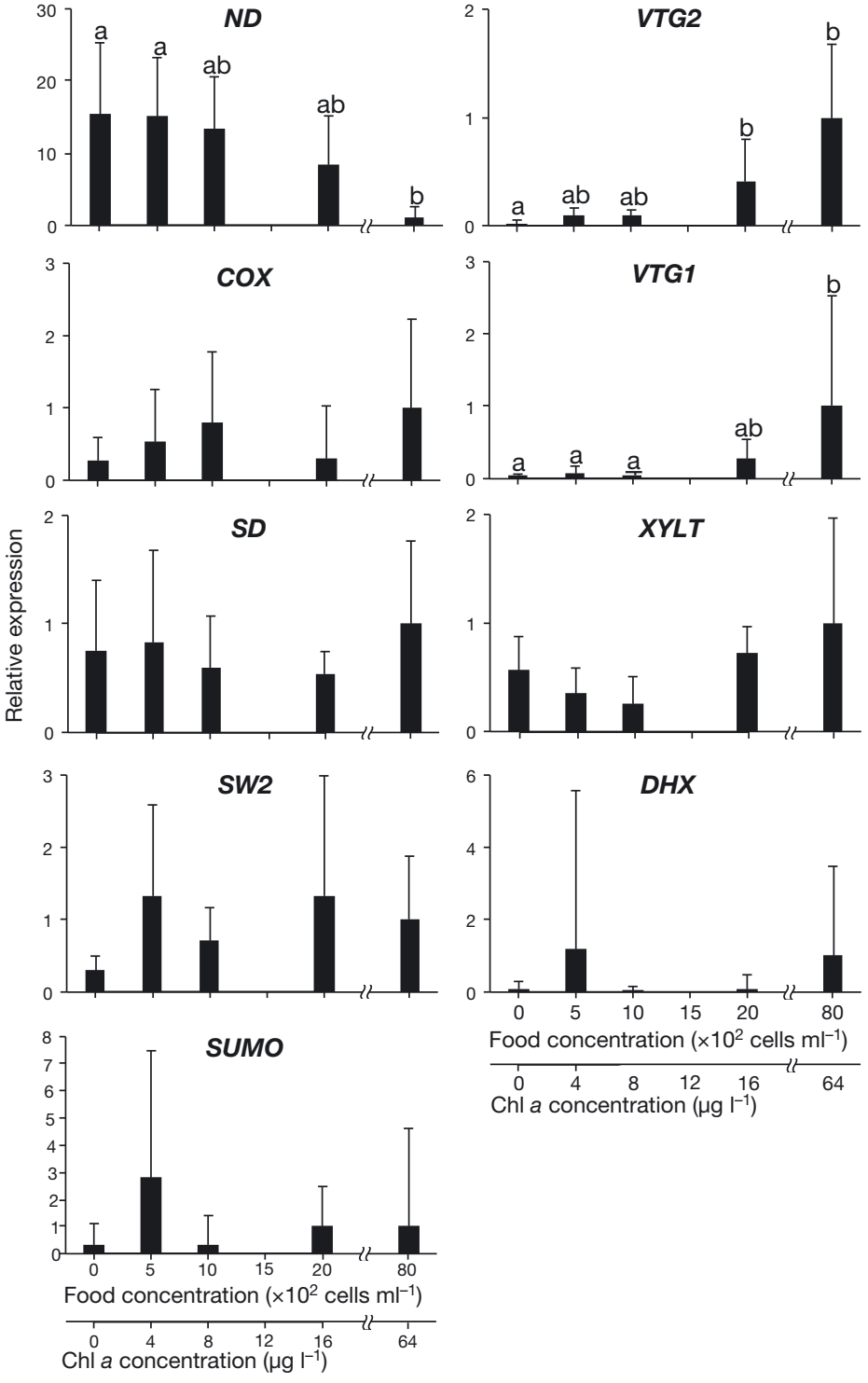

Fig. 3. Average relative gene expression at different food concentrations. Expression was measured by qRT-PCR and compared against values measured for individuals receiving the 8000 cells ml^{-1} treatment. Chlorophyll *a* concentration, which is the most common measure of phytoplankton abundance, is also shown along the *x*-axis. *ND*: NADH-dehydrogenase; *VTG2*: vitellogenin 2; *COX*: cytochrome *c* oxidase; *SD*: short-chain dehydrogenase; *VTG1*: vitellogenin 1; *XYLT*: xylosyltransferase; *SW2*: singed wings 2; *DHX*: ATP-dependent RNA helicase; *SUMO*: small ubiquitin-related modifier. The error bars show ± SD. Values with different letters (a and b) were significantly different among food concentrations (one-way ANOVA; Tukey's post-hoc test; $p < 0.05$). The absence of letters indicates no significant differences according to ANOVA

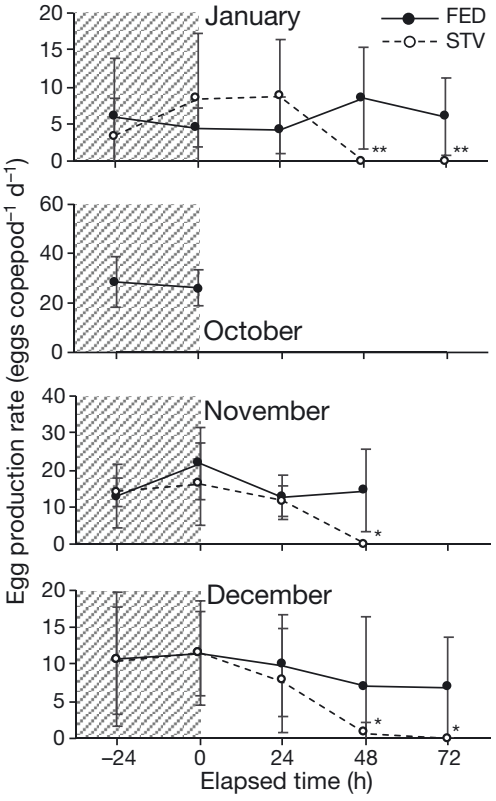

Fig. 4. Monthly average egg production rates of females during the acclimation (shaded) and starvation periods in the fed (FED) and starvation (STV) treatments. The error bars show ±SD. *p < 0.05; **p < 0.01 (Mann–Whitney *U*-test between FED and STV at each time point)

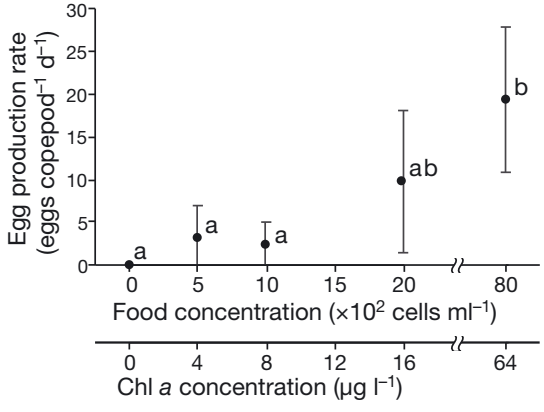

Fig. 5. Average egg production rates of females kept for 48 h in cultures with various food concentrations. Chlorophyll *a* concentration, is the most common value representing phytoplankton abundance, is also shown along the *x*-axis. The error bars show ±SD. Values with different letters (a and b) are significantly different among the food concentrations (one-way ANOVA; Tukey's post hoc test; p < 0.05)

ANOVA: $F_{4,70}$ = 6.223, p = 0.000; Tukey's test: p = 0.003–0.006). There were significant differences in EPR between the FED and STV groups in each fasting period at 48 and 72 h in January (*U*-test: p = 0.005 and 0.006), at 48 h in November (*U*-test: p = 0.017), and at 48 and 72 h in December (*U*-test: p = 0.046 and 0.048).

Overall, EPR tended to decline with decreasing food concentration (Fig. 5). There were significant differences in EPRs in the 0, 500, and 1000 cells ml^{-1} treatment groups compared with EPR in the 8000 cells ml^{-1} treatment group (ANOVA: $F_{4,14}$ = 4.950, p = 0.011; Tukey's test: p = 0.011, 0.037, and 0.026, respectively). No individual laid eggs at 0 cells ml^{-1}.

4. DISCUSSION

Our transcriptome analysis successfully identified 16 DEGs associated with starvation in female *Calanus sinicus*. There were 11 upregulated and 5 downregulated genes under the food-limited condition. Previous transcriptome studies on copepods have reported >100 DEGs correlated with environmental stresses such as high temperature (Lima & Willett 2017), acidification (Bailey et al. 2017), toxic algae (Roncalli et al. 2016), and heavy metal pollution (Wang et al. 2017). The number of DEGs found in our *C. sinicus* starvation experiment was lower than that reported in the aforementioned stress response studies; however, the studies are not directly comparable because different organisms and stress conditions were tested. A transcriptome study of *Drosophila melanogaster* identified fewer DEGs associated with starvation than that associated with other stress responses (Moskalev et al. 2015). A microarray analysis of *Calanus finmarchicus* targeting 1024 genes, which are hypothesized to be regulated by changes in the food environment, identified only 87 DEGs in individuals cultured for 7 d at low (500 cells ml^{-1}) and high food concentrations (5000 cells ml^{-1}) (Lenz et al. 2012). These differences in the numbers of DEGs associated with starvation or with other stresses corroborate the results of the present study; nonetheless, the number of DEGs identified in our study is relatively small. The late copepodite stages might be subjected to a food-limited environment for 12–16 h during the daytime as they migrate to deeper waters in the attempt to avoid predators (Runge 1980). Our culture conditions for complete fasting for 24 h, approximately twice as long as the diurnally experienced food limitation period associated with diel vertical migration, would have sufficed both temporally and quantitatively to affect the copepods' metabolism. The lack of an acclimation period before the RNA-Seq experiment,

however, may have accounted for the comparatively fewer DEGs found in this study. Different nutritional conditions and feeding histories may occur among individuals even within the same sample. These discrepancies could influence short-term gene expression responses to starvation, and result in the reduction of signal-to-noise ratio and power to detect differences between treatments. Our experiment with a 24-h fasting period could not reveal the complete physiological changes that occur during a prolonged starvation period. Thus, the DEGs identified in the present study might constitute only a part of the genes associated with the starvation response. Further RNA-Seq analyses based on individuals under various starvation conditions are needed to provide a comprehensive characterization of gene expression profiles related to the starvation response.

Various physiological changes have been reported as starvation responses in copepods. The general response of starved copepods, which can be observed using conventional methods, is a reduction in their metabolism (e.g. digestive enzyme activity, protein synthesis, and respiration rate). This reduction seems to appear within several days and inhibits somatic growth, development, and reproduction (Mayzaud 1976, Niehoff 2004). The energy conserved must be utilized to maintain basal metabolism when food is limited. The upregulated DEGs found in our RNA-Seq analysis implicated in energy metabolism were *ND*, *COX*, and *SD*. The upregulation of these genes by starvation indicated an increase in general cellular metabolism and mitochondrial electron transfer. The downregulated *VTG1*, *VTG2*, and *SW2* are probably associated with egg production or somatic growth. Vitellogenin (*VTG1* and *VTG2*) is a major egg-yolk precursor protein in oviparous species including crustaceans, insects, fish, and birds (Meusy 1980). Vitellogenesis, a central process in oocyte maturation, is the final pre-fertilization step wherein lipid yolk is formed, and it has been characterized in copepods (Niehoff 2004). In *D. melanogaster*, *SW2* indirectly controls an ecdysteroid molting hormone, ecdysone (Schwartz et al. 2004). In many insects, ecdysone is known as a key molecule for vitellogenin synthesis (Flanagan & Hagedorn 1977, Barchuk et al. 2002). Ecdysone is also known to control reproduction and embryogenesis in crustaceans (Mykles 2011). The present results suggest that in starved females, genes involved in growth-related and reproductive processes, which consume high amounts of energy and materials, are downregulated, whereas those genes related to processes involved in energy production to sustain basal metabolism during starvation are upregulated. *XYLT* is involved in the biosynthesis of the linkage region of glycosaminoglycan chains as part of proteoglycan biosynthesis (Stoolmiller et al. 1972). *DHX* is involved in the alteration of RNA secondary structure (Jankowsky 2011); this enzyme unwinds both RNA–RNA and RNA–DNA duplexes. *SUMO* is involved in cellular protein modification processes that participate in transcriptional regulation, nuclear transport, maintenance of genome integrity, and signal transduction (Johnson 2004). The associations between *XYLT*, *DHX*, and *SUMO* and starvation responses are unknown.

Robustness of a marker for starvation derives from its gene expression stability in the FED group and consistency in regulation under different fasting periods and food concentrations. Temporal expression changes in DEGs in response to environmental changes are classified either as short-impulse or long-sustained responses (Bar-Joseph et al. 2012). Short-impulse DEG responses are rapid upregulations or downregulations resulting from environmental changes that return to their initial expression. For example, in response to osmotic stress, the expression of the stress-responsive genes in yeast (e.g. mitogen-activated protein kinase and glucose-inactivated glycerol proton symporter) peaks at 10 min after stress induction but returns to the original expression level after 30 min (de Nadal et al. 2011). In contrast, long-sustained DEG responses maintain upregulation or downregulation as long as the environmental change is sustained. According to the qRT-PCR, the expression of *ND* was consistently upregulated and *VTG2* was downregulated with fasting periods (Fig. 2), and their expression was significantly different between the FED and STV groups after 6 h of fasting. Long-sustained DEG responses, like those responses for the expression of *ND* and *VTG2*, are robust markers under natural conditions. However, the response time of starvation may depend on the time at which the experiment was started. Feeding rhythms associated with intrinsic circadian rhythm have been suggested in copepods (Marcus 1986, Durbin et al. 1990). In the present study, all starvation experiments were initiated at night. There is a possibility that the food restriction at night may have a greater influence than the daytime food restriction, and the significant difference in expression shown after 6 h of fasting may depend on the start time of the experiment. Further studies on the response time of starvation would be required to determine whether this is the case. Both *ND* and *VTG2* tended to remain consistently regulated under

different food concentrations (Fig. 3). The expression of *ND* was upregulated and that of *VTG2* was downregulated with decreasing food concentrations. Therefore, relative differences in the expression of these genes may reflect the starvation period and the degree of starvation based on food availability. The other 7 candidates (*COX*, *VTG1*, *SD*, *XYLT*, *SW2*, *DHX*, and *SUMO*) lacked stability in the FED group and/or consistency in regulation under different fasting periods and food concentrations. The robustness of these candidates as markers for starvation is inferior to that of *ND* and *VTG2*.

ND is associated with oxidative phosphorylation in mitochondria. The upregulated expression of the genes involved in oxidative phosphorylation during starvation has been previously reported in several organisms including copepods (e.g. *C. finmarchicus*, *D. melanogaster*, mouse, and *Sparus aurata*; Lenz et al. 2012, Liu et al. 2014, Whitaker et al. 2014, Silva-Marrero et al. 2017). Because oxidative phosphorylation in mitochondria is essential for energy production, *ND* can be a marker associated with starvation not only in adult females but also in several earlier life stages. Downregulation of the expression of *VTG2* indicated a decrease in egg production. Consistent with previous studies on copepods (Dagg 1977, Runge 1984, Hirche & Bohrer 1987, Niehoff 2000), EPR apparently decreased to almost zero after 48 h of fasting (Fig. 4). An experiment involving the administration of radiolabeled food has shown that its intake affected egg synthesis in less than 8 h (Marshall & Orr 1972). In the present study, the expression of *VTG2* was significantly different after 6 h of fasting. Changes in the expression of *VTG2* appeared earlier than the observed decrease in EPR. Furthermore, the expression of *VTG2* was downregulated and EPR tended to decline with decreasing food concentration (Fig. 5). Although differences in age and gonadal development (Niehoff 2000, Niehoff & Runge 2003) might also affect *VTG* expression and EPR, the results of the present study suggest that *VTG2* expression is a reliable indicator of potential secondary productivity and that *VTG2* might be a female-specific marker associated with starvation. Because adult females control secondary production and population dynamics through egg production, evaluations of how food limitation affects them and identification of markers for starvation in females are particularly important.

In the present study, starved individuals clearly presented higher *ND* and lower *VTG2* expression than fed individuals. Differences in the expression of *ND* and *VTG2* indicated that the degree of starvation or food limitation in individuals could help assess potential reproductivity or predict EPR in *C. sinicus*. As *C. sinicus* is a key zooplankton species in the western North Pacific Ocean, evaluating the degree of its starvation in relation to food quantity and quality with these DEG markers may be used to assess secondary productivity and health of the trophic linkages in marine ecosystems.

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