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Effects of the oxylipin-producing diatom *Skeletonema marinoi* on gene expression levels of the calanoid copepod *Calanus sinicus*

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ABSTRACT

Diatoms are eukaryotic unicellular plants that constitute one of the major components of marine phytoplankton, comprising up to 40% of annual productivity at sea and representing 25% of global carbon-fixation. Diatoms have traditionally been considered a preferential food for zooplankton grazers such as copepods, but, in the last two decades, this beneficial role has been challenged after the discovery that many species of diatoms produce toxic metabolites, collectively termed oxylipins, that induce reproductive failure in zooplankton grazers. Diatoms are the dominant natural diet of *Calanus sinicus*, a cold-temperate calanoid copepod that supports secondary production of important fisheries in the shelf ecosystems of the Northwest Pacific Ocean, Yellow Sea, Sea of Japan and South China Sea. In this study, the effect of the oxylipin-producing diatom *Skeletonema marinoi* on *C. sinicus* has been evaluated by analyzing expression level changes of genes involved in defense and detoxification systems. Results show that *C. sinicus* is more resistant to a diet of this diatom species in terms of gene expression patterns, compared to the congeneric species *Calanus helgolandicus* which is an important constituent of the temperate waters of the Atlantic Ocean and northern Mediterranean Sea. These findings contribute to the better understanding of genetic and/or phenotypic flexibility of copepod species and their capabilities to cope with stress by identifying molecular markers (such as stress and detoxification genes) as biosensors for environmental perturbations (e.g. toxins and contaminants) affecting marine copepods.

1. Introduction

Calanus sinicus is a common cold-temperate calanoid copepod living in the shelf ecosystem of the Northwest Pacific Ocean, occurring in the Bohai Sea, the Yellow Sea, the Sea of Japan and the South China Sea (Hulsemann, 1994), where it supports secondary production of important fisheries, such as sardine and anchovy (Uye, 2000; Yang et al., 2014). In coastal waters off northern Taiwan, *C. sinicus* is a dominant species from winter to early spring, where it represents more than 50% of the winter copepod assemblage (Hwang et al., 2006). Its presence in the area is related to the southward intrusion of cold-water masses of the China Coastal Current during the northeast monsoon period, from November to March, which brings cold waters from the Yellow Sea and the East China Sea into the Taiwan Straits (Dur et al., 2007; Hwang and Wong, 2005; Tseng et al., 2013). Given its ecological importance, it is one of the target species in the China-GLOBEC program (Sun, 2005).

Several field studies have indicated that *C. sinicus* spawns continuously throughout the year in the Northwest Pacific Ocean, with maximum egg production rates during winter-early spring (Li et al., 2013; Uye, 2000; Wang et al., 2009; Zhang and Wong, 2013; Zhang et al., 2005, 2006), thus suggesting that the winter-spring diatom bloom could enhance copepod reproduction in this area. *C. sinicus* does in fact consume large quantities of diatoms as confirmed by a recent study on the gut contents of specimens collected during the winter season in northern Taiwan (The most abundant species found in the gut were *Thalassiothrix spp.*, *Chaetoceros spp.* and *Coscinodiscus spp.*; Chen et al., 2010). The study reported that diatoms represented more than 95% of the ingested food by *C. sinicus* females, thus confirming previous results of gut fluorescence analysis, according to which *C. sinicus* is considered a clear herbivorous species, although it can switch to omnivorous feeding when microzooplankton prey becomes available (Wang et al., 2009; Zhang et al., 2006).

It is known that several marine diatoms produce toxic polyunsaturated aldehydes (PUAs) and other products deriving from the oxidation of fatty acids (collectively termed oxylipins) that reduce reproductive success and induce larval malformations in several copepod species (Fontana et al., 2007; Ianora and Miralto, 2010 for a review).

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Recent studies have also shown that the oxylipin-producing diatom *Skeletonema marinoi* reduces the expression of several aldehyde-dehydrogenase genes and apoptosis-related genes in the copepod *Calanus helgolandicus* (Lauritano et al., 2011a,b, 2012a). *S. marinoi* also activated a generalized cellular stress response in *C. helgolandicus* by over-expressing genes of molecular chaperones and signal transduction pathways that protect the copepod from the damaging effects of a diet of this diatom species (Carotenuto et al., 2014).

To our knowledge, no studies have been performed so far to investigate the effect of oxylipin-producing diatoms on the reproduction of *C. sinicus*. Since diatoms dominate the diet of this copepod in the Northwest Pacific Ocean, and in particular in the water off northern Taiwan, it would be interesting to test whether such an inhibitory mechanism occurs in *C. sinicus* and if there are similarities with the congeneric species *C. helgolandicus*. The aim of the present study was, therefore, to investigate the response of stress-related genes in *C. sinicus* exposed to the oxylipin-producing diatom *S. marinoi* and compare the response of *C. sinicus* with *C. helgolandicus* (Lauritano et al., 2011a,b, 2012a). Although *S. marinoi* does not co-occur with *C. sinicus,* the congeneric oxyipin-producing *Skeletonema pacificum* does, and both species were considered the same species (*S. costatum*) until the genus was revised by Sarno et al. (2005). Both species produce oxylipins (Fontana, personal communication). In this study, genes have been selected with the attempt to include all the possible gene categories that can be involved in the response of *C. sinicus* to an oxylipin-producing diatom diet. We selected heat shock protein 70 (HSP70), genes involved in aldehyde detoxification (aldehyde dehydrogenase 2, aldehyde dehydrogenase 6, aldehyde dehydrogenase 8 and aldehyde dehydrogenase 9), free radical detoxification enzymes (i.e. catalase and superoxide dismutase) and enzymes involved in the metabolism of the scavenger molecule glutathione (glutathione synthase and glutathione S-transferase).

Since PUAs and oxylipins induce apoptosis and teratogenesis in the offspring of female copepods that have fed on diatoms for ≥5 d (Ianora et al., 2004), we therefore also determined the transcription level of the cell cycle and apoptosis regulatory 1 protein (CARP) and the cellular apoptosis susceptibility protein (CAS), both of which are involved in apoptosis (Brinkmann, 1998; Rishi et al., 2006). CARP is a novel cell growth regulator and CAS is necessary in the mitotic spindle checkpoint that ensures genomic stability during cell division.

Finally, we also selected the microtubule subunits, alpha- and beta-tubulin (Jordan, 2004), because previous studies indicated that a diet of *S. marinoi* affects their expression levels in *C. helgolandicus* females after 2 days of feeding by possibly reducing microtubule subunits, altering pronuclear migration, DNA replication and mitotic events (e.g. Buttino et al., 1999; Lauritano et al., 2011a).

Recently, the transcriptome of *C. sinicus* has been sequenced using both 454 pyrosequencing technology and Illumina Hiseq2000 thereby increasing the genomic resources available for this species (Ning et al., 2013; Yang et al., 2014) for ecological, physiological and population genetic studies (Minxiao et al., 2011). However, few stress-related and detoxification genes have been annotated in that study, making it difficult to investigate the response of the copepod to natural and/or human-derived toxins. Our findings, thus, will contribute to increase the number of useful genomic resources for this ecologically-relevant copepod species and will also contribute to identify molecular markers to be used as biosensors for environmental stressors (e.g. toxins and contaminants) affecting marine copepods.

2. Materials and methods

2.1. Copepod sampling

Zooplankton was collected in March and April 2013 in the East China Sea, 6 km away from Keelung City coast, east of Keelung Islet island (25° 11′ N; 121° 47′ E), with a Nansen net (200 μm mesh size) which was towed vertically from 30 meter depth to the surface. Specimens were transferred immediately to a 3 L plastic tank filled with surface seawater and bubbled air, and transported to the laboratory within 1 h after sampling. Healthy mature *C. sinicus* females were sorted using a stereomicroscope and placed in 250 ml beakers (20 animals/beaker) containing 0.45 μm mesh net filtered seawater (FSW) (33‰). Sorting was completed within 3–4 h after capture.

Two groups of 90 females each were incubated in 1 L bottles filled with FSW and acclimatized 24 h without food. After this period, one group of *C. sinicus* was fed with the control flagellate *Rhodomonas baltica* (8000 cells/ml, C content 1 mg Carbon/L) which does not produce oxylipins, and the other group was fed with the oxylipin-producing diatom *S. marinoi* (45,000 cells/ml, C content 1 mg Carbon/L). Bottles were maintained at 20 ± 2 °C and at a natural photoperiod. The culture medium was changed daily for both treatments.

After 2 and 5 days of feeding, 20 to 40 *C. sinicus* females from each diet were sorted and individually incubated in 60-ml crystallizing dishes without food for 3–4 h (at the same temperature and photoperiod as described above), to eliminate any algal residues in the gut and to avoid aspecific PCR (RT-qPCR) amplifications of the phytoplankton ingested. After 3–4 h, the bottom of the crystallizers was checked with the inverted microscope and fecal pellets were removed. When copepods did not produce any further pellets, 3 groups of 4–8 females each, were carefully transferred to 500 μl Trizol Reagent (Invitrogen), frozen in liquid nitrogen, and stored at −80 °C until shipping to the Stazione Zoologica Anton Dohrn of Naples for RNA extraction and RT-qPCR analysis. Wild control females (five groups of 8 females each) were also immediately isolated after sorting and incubated in crystallizers, to empty their guts, and were then treated as described above, for RNA extraction. Although we are aware that the experimental design can be considered pseudo-replication, the method revealed variability in gene expression between different groups of females exposed to the same diet.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from each copepod replicate according to Trizol manufacturer's protocol (Invitrogen). RNA quantity was assured by Nano-Drop (ND-1000 UV–vis spectrophotometer; NanoDrop Technologies) monitoring the absorbance at 260 nm; purity was determined by monitoring the 260/280 nm and 260/230 nm ratios using the same instrument. Both ratios were about 2.0. All samples were free from protein and organic solvents used during RNA extraction. RNA quality was evaluated by gel electrophoresis that showed intact RNA, with sharp ribosomal bands. 500 ng of each RNA was retro-transcribed into cDNA with the iScriptTM cDNA Synthesis Kit (BIORAD) following the manufacturer's instructions, using the GeneAmp PCR System 9700 (Perkin Elmer). The reaction was carried out in 20 μl final volume with 4 μl 5× iScript reaction mix, 1 μl iScript reverse transcriptase and H_2O. The mix was first incubated 5 min at 25 °C, followed by 30 min at 42 °C and finally heated to 85 °C for 5 min.

2.3. PCR (polymerase chain reaction) optimization

In order to perform gene expression analyses in *C. sinicus*, oligo that were already published for the copepod *C. helgolandicus* (Lauritano et al., 2011a,b) for both reference genes (RGs) and genes of interest (GOI) were optimized in a GeneAmp PCR System 9700 (Perkin Elmer). Reactions were carried out in 20 μl volume with 2 μl of 10× PCR reaction buffer Roche, 2 μl of 0.1% BSA, 2 μl of 10 × 2 mM dNTP, 0.8 μl of 5U/μl Taq Roche, 1 μl of 20 pmol/μl for each oligo, 1 μl template cDNA and nuclease free water to 20 μl. The PCR program consisted of a denaturation step at 95 °C for 3 min, 40 cycles at 95 °C for 30 s, 60 °C for 1 min and 72 °C for 30 s, and a final extension step at 72 °C for 7 min. Amplified PCR products were analyzed by 1.5% agarose gel electrophoresis in TBE buffer. In order to verify the correct assignment of amplicons to target genes, the resulting bands were excised from the gel and extracted according

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to the QIAquick Gel Extraction Kit protocol (QIAGEN) and the sequences were analyzed. Sequence reactions were obtained by BigDye Terminator Cycle Sequencing Technology (Applied Biosystems) and purified using the Agencourt CleanSEQ Dye terminator removal Kit (Agencourt Bioscience Corporation) in automation by the robotic station Biomek FX (Beckman Coulter). Products were analyzed on the Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems). The identity of each sequence was confirmed using blastn (nucleotide sequence *vs* nucleotide collection) in the bioinformatics tool BLAST (Basic local alignment search tool) and the best hit species and NCBI accession numbers for each gene are reported in Table 1.

2.4. Best reference gene (RG) assessment

In order to analyze expression levels of specific GOI, a panel of putative reference genes (RGs) was first screened to find the most stable genes in the new species *C. sinicus* in both natural and experimental (feeding experiments in the laboratory) conditions. The selected genes were: elongation factor 1a (EFA), adenosine 3-phosphate synthase (ATPs), histone 3 (HIST), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal units (18S, S7, S20), ubiquitin (UBI) and beta actin (ACT). Three different algorithms were utilized to identify the best RGs in our experimental design: BestKeeper (Pfaffl et al., 2004), geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004).

2.5. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Expression level analyses were then performed for specific GOIs: heat-shock proteins 40 and 70 (HSP40, HSP70), cytochrome P450-4 (CYP4), catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), glutathione synthase (GSH-S), six aldehyde dehydrogenases (ALDH2, ALDH3, ALDH6, ALDH7, ALDH8, ALDH9), inhibitor of apoptosis protein (IAP), cell cycle and apoptosis regulatory 1 protein (CARP), cellular apoptosis susceptibility protein (CAS) and alpha and beta tubulins (ATUB and BTUB, respectively). Serial dilutions of cDNA were used to determine both RGs and GOI primer reaction efficiency and correlation factor (see Table 1), generating standard curves with five dilution points by using the cycle threshold (Ct) value versus the logarithm of each dilution factor and using the equation $E = 10^{-1/\text{slope}}$. Genes, both RGs and GOI, with low oligo efficiency (lower than 75%) were discarded (i.e. S7, ALDH3, ALDH7, HSP40, CYP and IAP). RT-qPCR was performed in MicroAmp Optical 384-Well reaction plate (Applied Biosystem, Foster City, CA) with optical adhesive covers (Applied Biosystem) in a Viia7 real-time PCR system (Applied Biosystem) and using the fluorescent dye Fast Start SYBR Green Master Mix (Roche, Indianapolis, IN). The PCR volume for each sample was 10 μl, with 5 μl of Fast Start SYBR Green Master Mix, 1 μl of cDNA template (1:50 template dilution) and 0.7 pmol/ml for each oligo. The RT-qPCR thermal profile was obtained using the following procedure: 95 °C for 10 min, 40 times 95 °C for 15 s, and 60 °C for 1 min, followed by one final step of 72 °C for 5 min. The program was set to reveal the melting curve of each amplicon from 60 to 95 °C, and read every 0.5 °C. Only a single peak was identified in the melting-curve analyses of all genes, confirming a gene-specific amplification and the absence of primer-dimers. All RT-qPCR reactions were carried out in triplicate to capture intra-assay variability. Each assay included three no-template negative controls (NTC) for each primer pair. To study expression levels for each GOI relative to the most stable RGs (S20, EFA and UBI), we used the REST tool (Relative Expression Software Tool) (Pfaffl et al., 2002). Female copepods collected during the same sampling and starved for less than 24 h to eliminate any algal deposit from the gut, were used as controls. Statistical analysis was performed using both the Randomization test from REST and the GraphPad Prim statistic software, V4.00 (GraphPad Software).

3. Results

3.1. Best reference genes (RGs) assessment

In order to analyze expression levels of specific genes of interest (GOI), a panel of putative reference genes (RGs), necessary to normalize reverse transcription-quantitative polymerase chain reaction (RT-qPCR) data, was first screened to find the most stable genes in the experimental conditions. Raw Ct data of potential RGs are reported in Fig. 1. Values are similar between genes, except for the ribosomal RNA 18S that are between 15 and 20. 18S is very highly expressed with a threshold cycle that was not similar to the other RGs and genes of interest. Hence, we discarded it as a reference (Kozera and Rapacz, 2013). According to the mathematical approach of BestKeeper (Pfaffl et al., 2004), RG expression stability considers the standard deviation of the Ct values

Table 1
Table 1 lists selected reference and genes of interest abbreviation names, best blast hit GenBank species and accession numbers (Acc. no.), sequences of forward and reverse primers, amplicon length (L), oligo efficiencies (E) and correlation factor (R). *Calanus* is abbreviated as *C.* and *Pseudodiaptomus* as *P.*

Gene name abb.	Acc. no.	Top hit species	Forward primer sequence 5′-3′	Reverse primer sequence 5′-3′	L	E	R
EFA	HQ270534	*C. helgolandicus*	GACAAGCCCCTCAGACTTCC	GGAGAGACTCGTGGTGCATC	172	90%	0.9995
ATPs	HQ270527	*C. helgolandicus*	CTCCATCACTGACGGACAGATC	TCAAGCTTCATGGAACCAGC	150	95%	0.9973
HIST	HQ270530	*C. helgolandicus*	GAGGAGTGAAGAAGCCCCAC	TGAAGTCCTGAGCAATCTCCC	137	99%	0.9979
18S	GU969174	*C. sinicus*	GAAACCAAAGCATTTGGGTTC	GCTATCAATCTGTCAATCCTTCC	164	91%	0.998
GAPDH	HQ270535	*C. helgolandicus*	ATCTTTGATGCCAAGGCTGG	GTCCTTGCCCTGCATGAAG	126	100%	0.9925
S20	HQ270531	*C. helgolandicus*	CGTAAGACTCCTTGTGGTGAGG	GAAGTGATCTGCTTCACGATCTC	113	94%	0.9902
UBI	HQ270536	*C. helgolandicus*	GCAAGACCATCACCCTTGAG	CAGCGAAAGATCAACCTCTG	113	94%	0.9951
ACT	HQ270533	*C. helgolandicus*	GGCACCACACTTTCTACAACG	GTTGAAGGTCTCGAACATGATC	131	100%	0.99
ATUB	HQ270529	*C. helgolandicus*	ACAGCTTCTCCACCTTCTTCTC	GTTGTTGGCGGCATCCTC	168	95%	0.9926
BTUB	HQ270528	*C. helgolandicus*	GGATTTCAGCTGACCCACTC	GTCTCATCAGTATTTTCCACCAG	205	100%	0.9881
ALDH2	JF825506	*C. helgolandicus*	GGACAAGGCAGATGTCAACAA	ATAGGGTTTGCCATTGTCAAG	181	94%	0.9971
ALDH6	JF825508	*C. helgolandicus*	GAGCAGTGCTGCAGCAACAC	GGAACATCCAGAGGGGGATC	164	100%	0.9927
ALDH8	JF825510	*C. helgolandicus*	CTGGAGGAGTTTGCAGTGG	GCCAGCCACACCAATAGG	198	100%	0.9964
ALDH9	JF825511	*C. helgolandicus*	GGAAAACCAATCTGGGAAGC	CAAAGGGTAGTTCCAGGCTC	183	100%	0.9975
GST	JF825513	*C. helgolandicus*	CAACCCCCAGCACACTGTG	GGATAGACACAATCACCCATCC	210	98%	0.9987
GSH-S	JF825516	*C. helgolandicus*	GAGAAGGCAAAGGACTATGCTC	GGCAACCTTGTGCATCAAC	180	100%	0.9953
CAT	JF825517	*C. helgolandicus*	TGTACATGCAAAGGGAGCTG	GGTGTCTGTTTGCCCACTTT	104	100%	0.9982
SOD	JF825518	*C. helgolandicus*	GGAGATCTTGGCAATGTTCAG	CAGTAGCCTTGCTCAGTTCATG	166	100%	0.9964
CAS	JF825520	*C. helgolandicus*	CTACAACCACTACCTGTTCGAGT	CAGGGACATGATCTGGAACAC	169	100%	0.9777
CARP	JF825519	*C. helgolandicus*	GCCAAGAGTGGGAAGTTTGAC	GAACATTTCATTGAACAATTCTGC	126	100%	0.9959
HSP70	JX624124	*P. annandalei*	CTTCGTTTGGTATCCATGTTGGTA	CTCTGTGTCCTGGTAGGCGAC	130	100%	0.9976

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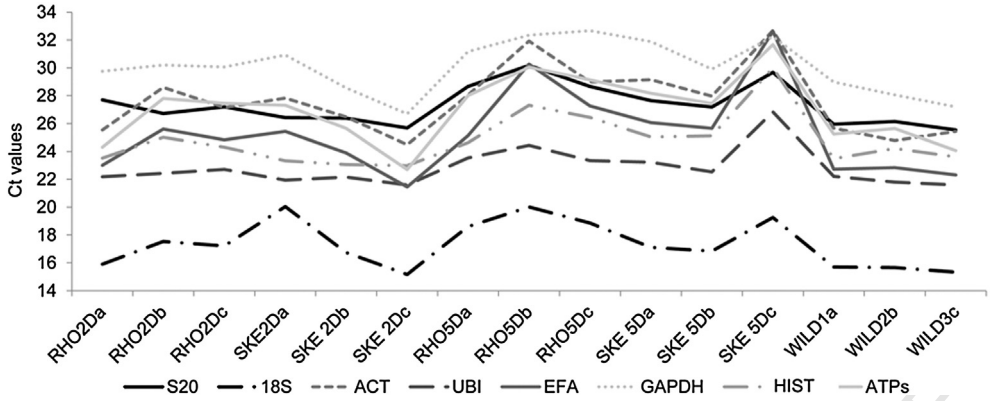

Fig. 1. The threshold cycle values. The threshold cycle (Ct) values (ordinates) obtained for all candidate reference genes (RGs) in the copepod *Calanus sinicus* during the feeding experiments with *Rhodomonas baltica* and *Skeletonema marinoi* diets for 2 or 5 days, or in copepods sampled and harvested for 24 h without food (WILD) (abscissa). Each curve represents the degree of stability of Ct values for each RG.

(Fig. 2a). The most stable RG has a standard deviation (SD) lower than 1 and in our case, this was UBI. GeNorm analysis (Vandesompele et al., 2002) considers as best reference gene couple those genes with the lowest expression stability (M); in this study, the two most stable genes were UBI and S20 (Fig. 2b). According to the third statistical approach utilized, NormFinder, the best reference gene was EFA, also in this case the gene with the lowest stability value (Fig. 2c). A synopsis of the results is summarized in Table 2. Although BestKeeper and GeNorm approaches agreed that the best RG was UBI, the three types of approaches gave different results, depending on the software, hence we used as best reference genes the ones that ranked as first for each software: UBI, S20 and EFA.

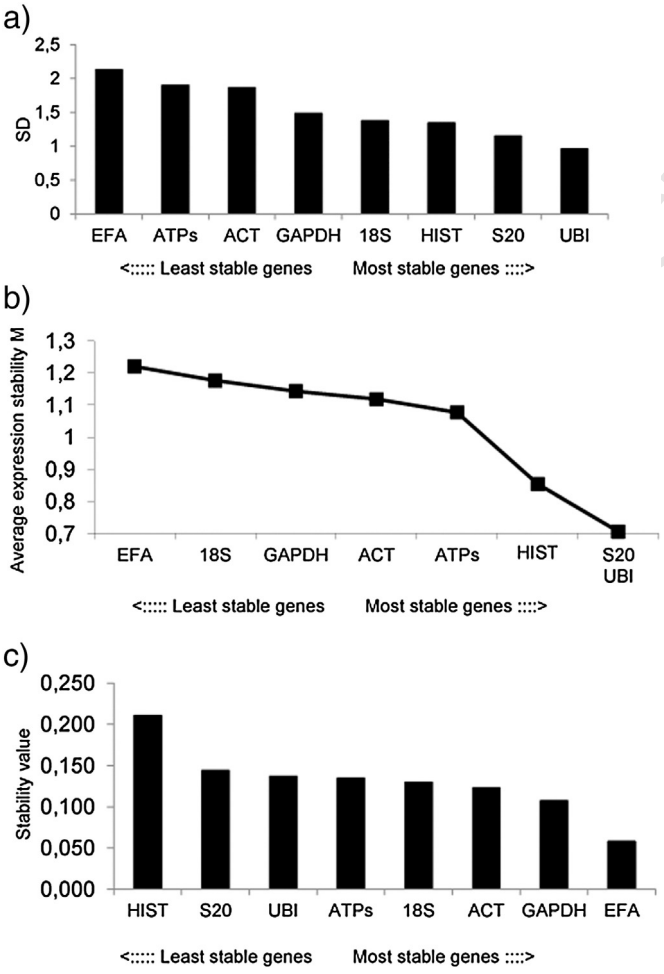

Fig. 2. Reference gene assessment. Ranking of the best reference genes (RGs) obtained with BestKeeper, GeNorm and NormFinder. (a) The best reference genes have the lowest Ct value-standard deviation for BestKeeper, (b) the lowest average expression stability (M) for geNorm and (c) the lowest stability value for NormFinder analysis (as indicated by the arrows).

3.2. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Fig. 3 shows differential gene expression in the copepod *C. sinicus* after the ingestion of the diatom *S. marinoi* for two or five days. Feeding upon a diet based on *S. marinoi* for two days induced the down-regulation of one microtubule subunit, beta tubulin (BTUB) and one out of four aldehyde dehydrogenases, ALDH6 ($p < 0.001$ for both genes). After 2 days of ingestion of *S. marinoi* the antioxidant enzyme CAT was also down-regulated ($p < 0.001$). HSP70 levels decreased after both 2 and 5 days ($p < 0.001$ only after 2 days).

After 5 days of ingestion of *S. marinoi*, most of the analyzed genes were up-regulated. In particular, all the four aldehyde dehydrogenases increased their expression levels, but due to high variability between replicates the results were statistically significant only for ALDH2 and ALDH9 ($p < 0.05$). All genes related to antioxidant activity and detoxification of free radicals, GST, GSH-S, CAT and SOD increased their expression levels after 5 days of ingestion ($p < 0.05$ for CAT and GSH-S). Finally, BTUB that was down-regulated after 2 days of feeding on *S. marinoi* and was up-regulated after 5 days, suggesting a cellular restoring of the missing microtubule subunit. The apoptosis-related genes CAS and CARP did not show significant results, indicating the absence of a death-related signal after the ingestion of the oxylipin producing diatom for two and five days.

Table 2
Ranking of the best reference genes as given by BestKeeper, NormFinder and Genorm analyses.

Ranking	Bestkeeper	Normfinder	Genorm
1	**UBI**	**EFA**	**UBI/S20**
2	S20	GAPDH	
3	HIST	ACT	HIST
4	18S	18S	ATPs
5	GAPDH	ATPs	ACT
6	ACT	UBI	GAPDH
7	ATPs	S20	18S
8	EFA	HIST	EFA

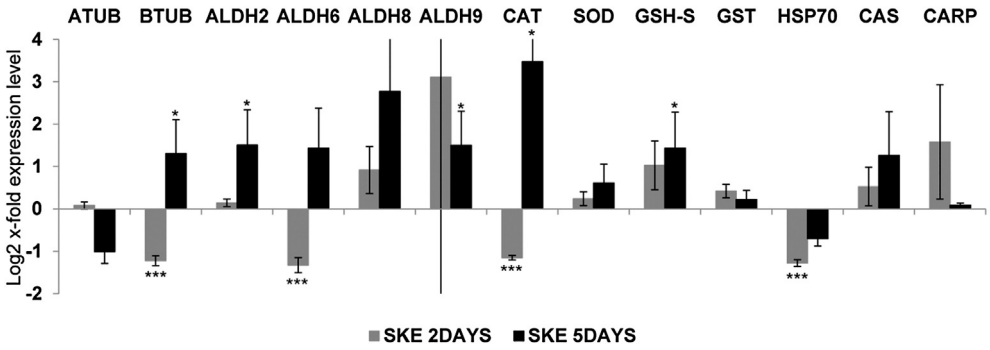

Fig. 3. Genes of interest expression levels. Relative gene expression levels of genes involved in generic stress responses, aldehyde dehydrogenases (ALDH) and apoptosis regulation in the copepod *C. sinicus* feeding on the oxylipin-producing diatom *S. marinoi* for 2 or 5 days compared to the control (represented in the figure by *x*-axis). Two days of feeding are represented in the figure by histograms in gray and five days in black. The data are normalized with the three best RGs, UBI, S20 and EFA.

4. Discussion

Our results indicate that after 2 days of feeding on the diatom *S. marinoi*, there was a light impairment of the stress and antioxidant defense systems in the copepod *C. sinicus*, with a reduction of CAT and HSP70 expression levels. In addition, there was the down-regulation of one of the microtubule subunits, BTUB, possibly reducing microtubule filament formation, and of one out of six aldehyde dehydrogenases analyzed. However, after 5 days of feeding, there was a significant increase in the expression of BTUB, ALDH2, ALDH9, CAT and GSH-S, suggesting a possible restoration of the damaged proteins and the activation of a protecting and antioxidant response.

These findings differ from previous studies showing that two days of feeding on *S. marinoi* were sufficient to inhibit a series of genes of interest in the congeneric species *C. helgolandicus* from the Mediterranean Sea, with a strong down-regulation of at least 50% of the analyzed genes (ALDH6, ALDH8 and ALDH9, cellular apoptosis susceptibility and inhibitor of apoptosis proteins, one heat shock protein, and alpha- and beta-tubulins) (Lauritano et al., 2012a, summarized in Fig. 4). However, different responses were observed in various *C. helgolandicus* populations (Adriatic Sea, Swedish western coast and English Channel) due to their differing tolerance to toxic metabolites (Lauritano et al., 2012a). The Mediterranean population was the more susceptible to the toxic diet compared to the others, showing the down-regulation of most all the analyzed genes. On the contrary, the other populations (especially the North Atlantic Swedish population) were able to activate both antioxidant and stress-related genes (e.g. ALDHs, CAT and HSPs) in order to possibly detoxify the toxic algal secondary metabolites. All in all, our results suggest that defense responses change depending on the species/populations studied and on their detoxification capacities.

In recent years, numerous studies have focused on the effects of stressors on aquatic organisms, showing that responses to toxicants tend to be species-specific and may also be due to pre-adaptation to a given xenobiotic (Colin and Dam, 2007; Lauritano et al., 2012a; Sotka

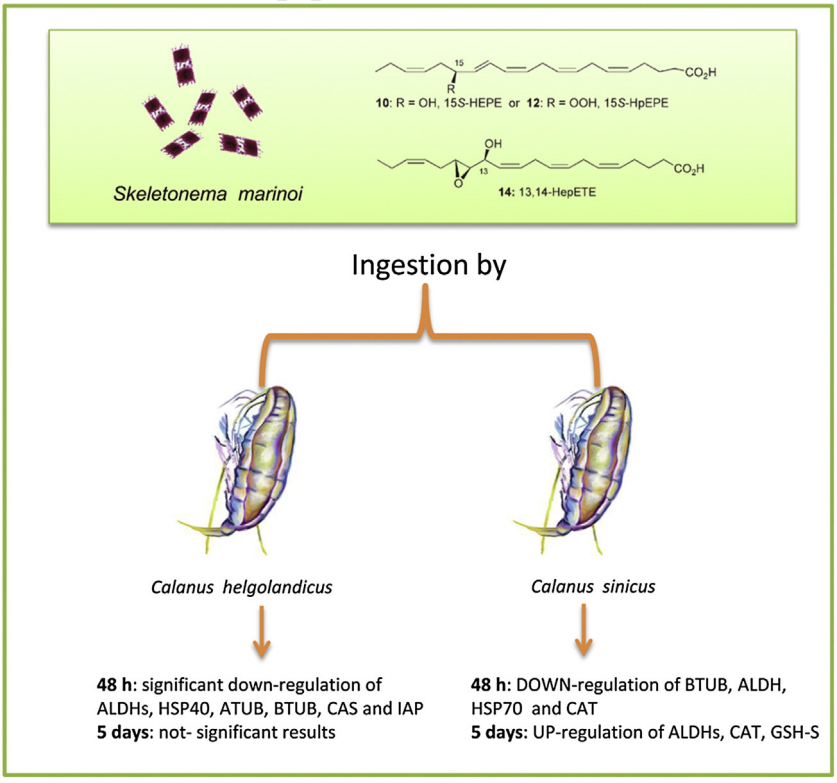

Fig. 4. Synopsis. Synopsis of the results obtained in this study and comparison with previous results obtained in published works on the copepod *Calanus helgolandicus*.

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and Whalen, 2008; Vidal and Horne, 2003). Heat shock proteins, antioxidant and ROS detoxification enzymes have been analyzed in copepods exposed to various environmental contaminants, such as heavy metals, endocrine disruptor chemicals and hydrocarbons, and the oxylipin-producing diatoms *S. marinoi* and *Chaetoceros socialis* (see review Lauritano et al., 2012b). The data indicate high inter- and intra-species variability in copepod responses, depending on the type of stressor tested, the concentration and exposure time, and the enzyme isoform studied.

Genetic differentiation and/or phenotypic flexibility have already been shown to be responsible for a species' ability to cope with environmental constraints (Blanckenhorn, 1997; Lardies and Bozinovic, 2008).

In a global change perspective, future physical–chemical variations may favor some species compared to others. The Mediterranean (Adriatic) *C. helgolandicus* population, for example, seems to be a species with fewer possibilities to cope with unfavorable constraints, such as oxylipins, while other species (*C. sinicus*) or populations of the same species *C. helgolandicus* (i.e. Swedish western coast and English channel) have greater possibilities to cope and survive. Copepods support 70–90% of zooplankton biomass (Kiorboe, 2011) and exert key roles in marine functioning (Rivkin and Legendre, 2001) and biogeochemistry (Mauchline, 1998); hence, it is essential to understand the effects of antipredatory metabolites and how plankton chemical interactions can shape biodiversity and ecological functioning from the community to the cellular scale. Our findings contribute to the better understanding of plankton chemical interactions and in the identification of molecular markers (such as stress and detoxification genes) as biosensors for environmental stressors (e.g. toxins and contaminants) affecting marine copepods.

5. Uncited references

Buttino et al., 2008
Kang et al., 2011
Li et al., 2006

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