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Gene expression patterns and stress response in the copepod *Calanus helgolandicus*

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Abstract

Diatoms and dinoflagellates are dominant photosynthetic organisms in the world's oceans and are considered essential in the transfer of energy through marine food chains. However, these unicellular organisms produce secondary metabolites such as products deriving from the oxidation of fatty acids collectively termed oxylipins (including polyunsaturated aldehydes or PUAs; by diatoms) or potent neurotoxins (brevetoxins; by dinoflagellates). It is often assumed that harmful algae toxins are grazing deterrents to discourage zooplankton grazers from eating these algae. Some laboratory studies have suggested that some toxic algae are either not eaten by various grazers or that grazers ingesting toxic algae suffer adverse effects such as reduced feeding rates, diminished reproductive success, behavioral modification or increased mortality (Cohen et al., 2007, Kubanek et al., 2007; Prince et al., 2006).

The aim of this thesis was to study in the copepod *Calanus helgolandicus* the effects of toxic diets at the molecular level. Expression level analyses by the sensible technique reverse transcription-quantitative polymerase chain reaction (RT-qPCR) allowed the study of specific genes of interest (GOI) which are known to have a primary role in generic stress responses, defense systems (e.g. aldehyde, free fatty acid and free radical detoxification) or apoptosis regulation in other organisms, from humans to marine organisms (Bouraoui, *et al.*, 2009; Einsporn, *et al.*, 2009; Hasselberg, *et al.*, 2004; Kim, *et al.*, 2008; Olsvik, *et al.*, 2009; Salazar-Medina, *et al.*, 2010; Snyder, 2000; Vasiliou, *et al.*, 2004; Wan, *et al.*, 2011). The GOI analyzed were two heat shock proteins (HSP70 and HSP40), six Aldehyde dehydrogenases (ALDH2, ALDH3, ALDH6, ALDH7, ALDH8, ALDH9), Cytochrome P450-4 (CYP4), Catalase (CAT), Superoxide Dismutase (SOD), Glutathione S-Transferase (GST), Glutathione Synthase (GSH-S), Inhibitor of Apoptosis Protein (IAP), Cell Cycle and Apoptosis Regulatory 1

Protein (CARP), Cellular Apoptosis Susceptibility Protein (CAS), actin (ACT) and Alpha and Beta tubulins (ATUB and BTUB). These GOI were analyzed in various experimental conditions: copepods exposed to algae which produce or do not produce toxic metabolites, including dinoflagellates (*Prorocentrum minimum*, *Rhodomonas baltica* or *Karenia brevis*) and diatoms (*Chaetoceros socialis* and *Skeletonema marinoi*), during field or laboratory experiments. In addition, the effect of the oxylipin producing diatom *Skeletonema marinoi* has been tested on two different *C. helgolandicus* populations: the Mediterranean population collected in the Adriatic Sea and the Atlantic population collected in the English Channel.

According to the results obtained, expression levels of the specific GOI changed depending on the tested algae, times of exposure, copepod population analyzed and field/laboratory experiments. Gene expression level patterns in the different experimental conditions tested may help to understand the copepod response to stressful conditions. The identification of new genes, for example using cDNA libraries or new generation sequencing, and the application of new tools, such as functional proteomic approaches, may allow for a more comprehensive overview of how copepods respond to specific stressors in the laboratory, but also to predict the response under natural environmental conditions and the effects of these responses on higher trophic levels.

Chapter 1: Introduction

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1.1 Gene expression patterns and stress response in marine organisms

Aquatic organisms are constantly exposed to environmental stimuli and natural and/or dissolved anthropogenic variables/compounds, including both physical (e.g. cold, heat and osmotic condition) and chemical (e.g. endocrine disruptor chemicals and hydrocarbons) stressors. Organisms may react to these stressors by activating a series of cellular defense systems that can be summarized as "first and second lines of defense" (Kozlowsky-Suzuki, *et al.*, 2009; Luckenbach, Epel, 2008). A synopsis of the most important enzymes and proteins involved in defense systems and stress responses is reported in table 1.1. The first line of defense is a multixenobiotic resistance system (MXR), also known as multidrug resistance system (Sarkadi, *et al.*, 2006), involved in the efflux of a large number of structurally and functionally diverse, moderately hydrophobic compounds, including anthropogenic pollutants and natural toxins (Bard, *et al.*, 2002; Kozlowsky-Suzuki, *et al.*, 2009). MXR are plasma membrane proteins (e.g. P-glycoprotein, P-gp), members of the adenosine triphosphate (ATP)-binding cassette (ABC) super family of energy-dependent efflux protein pumps, that act as active efflux pumps leading to lower intracellular accumulation of xenobiotic substrates (Kozlowsky-Suzuki, *et al.*, 2009; Luckenbach, Epel, 2008). MXRs have been detected in a plethora of marine organisms including sponges, mussels, oysters, crabs, worms, sea stars, clams and fishes (Bard, 2000; Epel, *et al.*, 2006; Faria, *et al.*, 2011; Minier, *et al.*, 2008; Roepke, *et al.*, 2006; Sauerborn, *et al.*, 2004) exposed to different stress conditions: e.g. reef coral *Montastraea franksi* exposed to copper (Venn, *et al.*, 2009) and fish *Mugilogobius abei* exposed to hydrocarbons, pesticides and heavy metals (He, *et al.*, 2011). In some cases, contaminants have been observed to inhibit MXRs, even if it is

not clear if such compounds are powerful MXR inhibitors or if inhibition is a result of saturation of MXR proteins by numerous substrates (Smital, *et al.*, 2004). In copepods, MXR proteins have only been studied in the sea lice *Lepeophtheirus salmonis* (*L. salmonis*) exposed to emamectin benzoate (EMB), the most effective drug administered to salmon against *L. salmonis* infestation (Tribble, *et al.*, 2007).

The "second line of defense", via metabolic enzymes, essentially consists in two phases, catalyzed by Phase I and Phase II enzymes, the purpose of which is to facilitate elimination of compounds from the body (Kozlowsky-Suzuki, *et al.*, 2009). Phase I reactions can involve oxidation, reduction, hydrolysis, hydration and dehalogenation of these compounds. The most common reaction is to oxidize them by converting a C-H bond to a C-OH, which is the reaction site for successive possible detoxification reactions. Phase I enzymes are mainly the cytochrome P450 (CYP450) supergene enzyme family that generally constitute the first enzymatic defense against foreign compounds. Phase II reactions generally follow those of Phase I and consist in conjugation reactions that render the substrate water-soluble thereby facilitating elimination from the cell. Examples of conjugation enzymes are glutathione S-transferase, glucuronyl transferase and sulphotransferase which transfer a polar compound (glutathione, glucuronic acid and sulphate, respectively) to the product obtained from phase I reactions. Both lines of defense have been found in many organisms, from prokaryotes to eukaryotes (van Straalen, Roelofs, 2006), even if the isoform number of each enzyme differs between species. Cytochrome P450 enzyme and glutathione S-transferase alterations in marine organisms have been found after exposure to many typical pollutants that are continuously released into the environment: polycyclic aromatic hydrocarbons, heavy metals (e.g. Pb and Cu) and endocrine disruptor chemicals (e.g. benzo[a]pyrene and alkylphenols) (Bouraoui, *et al.*, 2009;

Einsporn, *et al.*, 2009; Hasselberg, *et al.*, 2004; Salazar-Medina, *et al.*, 2010; Snyder, 2000; Wan, *et al.*, 2011).

Acronime and gene name	**Gene function**
First line of defense	
Multixenobiotic resistance system (MXR) (i.e. P-gp)	Efflux of compounds, including anthropogenic pollutants and natural toxins
Second line of defense	
Phase I:	
Cytochrome P450 (CYP450) enzymes	The most common reaction is to oxidize compounds by converting a C-H bond to a C-OH, which is the reaction site for successive possible detoxification reactions
Phase II:	
Glutathione S-transferases (GSTs)	The transfer of a polar compound, glutathione, to the product obtained from phase I reactions.
Glucuronyl transferase	The transfer of glucuronic acid to the product obtained from phase I reactions.
Sulphotransferase	The transfer of sulphate to the product obtained from phase I reactions.
Free radical detoxification	
Superoxide dismutase	Superoxide radicals ($*O^{2-}$) are dismutated into hydrogen peroxide (H_2O_2)
Catalase	H_2O_2 is further converted into water and divalent oxygen (O_2)
Glutathione peroxidase	H_2O_2 is further converted into water and divalent oxygen (O_2)
Glutathione, carotenes (vit A), ascorbic acid (vit C) and tocopherols (vit E)	Antioxidant molecules
Heat shock proteins (HSPs)	Molecular chaperones that can be involved in protein folding and unfolding, and degradation of mis-folded or aggregated proteins
Aldehyde deydrogenases (ALDHs)	ALDHs catalyze the oxidation of endogenous and exogenous aldehydes into their corresponding carboxylic acids.

Table 1.1. Synopsis of the most important enzymes and proteins involved in defence systems and stress response.

An important source of stress for most organisms is the increase of reactive or toxic intermediates and free radicals such as superoxide anion, hydroxyl and nitric oxide radicals, and peroxynitrite. In low quantities, these are rapidly converted to less reactive forms, but, when present in abnormally high quantities, these free radicals can be very damaging to DNA, RNA and proteins. To detoxify these deleterious molecules, cells possess their own free radical detoxification enzymes such as catalase, superoxide dismutase and glutathione peroxidase, and scavenger molecules such as glutathione, carotenes (vit A), ascorbic acid (vit C) and tocopherols (vit E). Superoxide radicals ($*O^{2-}$) are dismutated into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 is further converted into water and divalent oxygen (O_2), benign molecules, by catalase (CAT) and glutathione peroxidase, while the scavenger molecules act as cellular antioxidants preventing damage by reactive oxygen species (ROS). Proteins damaged by ROS can be denatured or form cross-links. Their refolding is driven by heat shock proteins (HSPs) that are activated in response to various environmental stress factors such as heat, hypoxia, UV radiation, and chemical exposure (Bierkens, 2000; Feder, Hofmann, 1999; Tartarotti, Torres, 2009). HSPs are molecular chaperones that can be involved in protein folding and unfolding, and degradation of mis-folded or aggregated proteins (Sorensen, *et al.*, 2003). Temperature and salinity variations, EDC and heavy metal exposure have been found to induce significant changes in HSP activities in various marine organisms in laboratory and field conditions (Franzellitti, *et al.*, 2010; Monari, *et al.*, 2011; Ribecco, *et al.*, 2011; Roccheri, *et al.*, 2004) and, in some cases, the effects were reversible (Roccheri, *et al.*, 2004). Catalase and superoxide dismutase have been analyzed in marine invertebrates mainly after heavy metal stress exposure (Buffet, *et al.*, 2011; Main, *et al.*, 2010). Few studies have been performed on stress responses in copepods, an important class of crustaceans that is critical in the

transfer of energy from primary producers to higher trophic levels in marine food web (Figure 1).

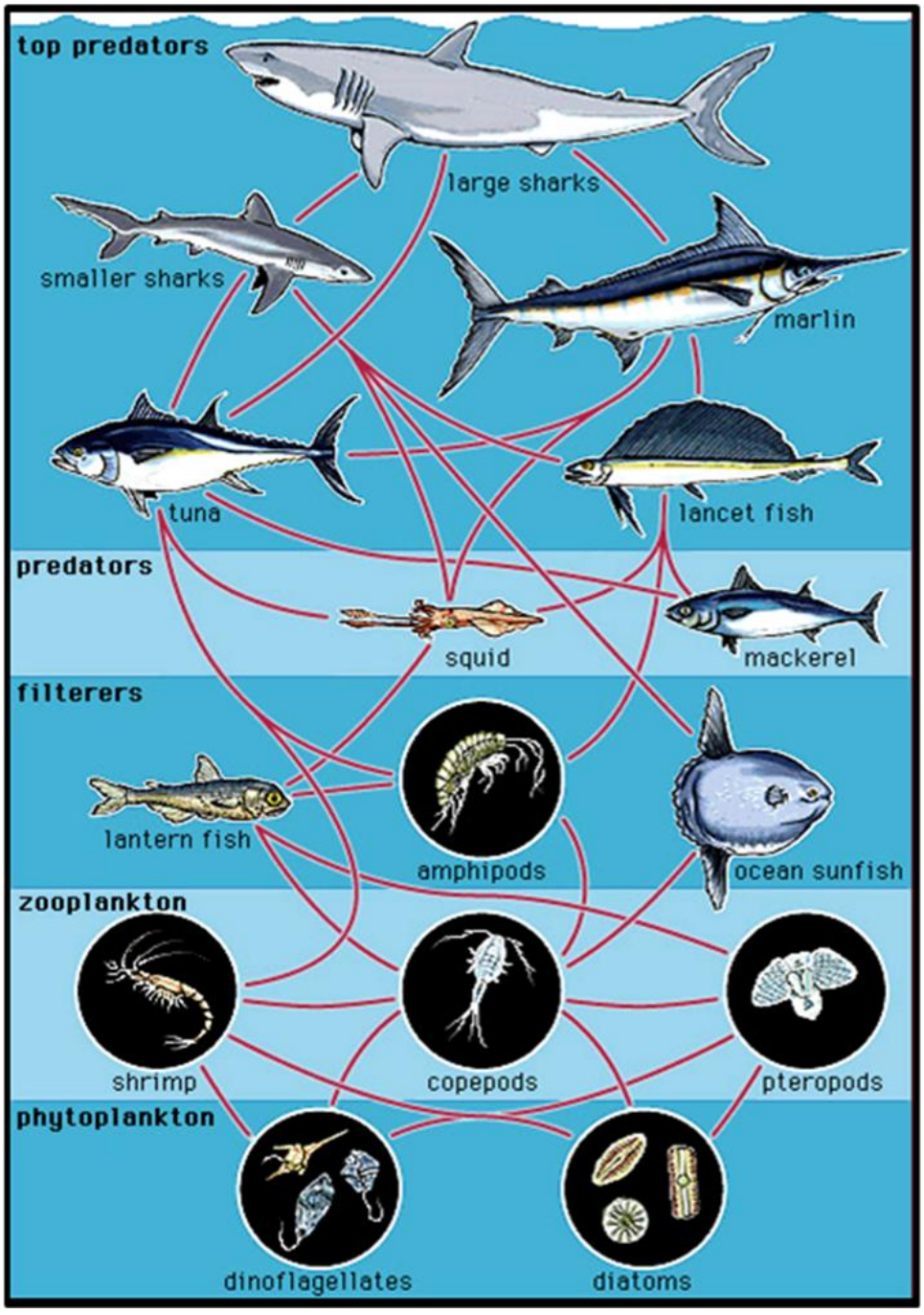


Figure 1.1. Marine food chain from Encyclopaedia Britannica 1996.

1.2 Copepods

Copepods are a group of small crustaceans that are critical components of the world's freshwater and marine ecosystems. Their name "copepod" derives from the greek word "kope", meaning "oar", and "podos", meaning "foot" (Mauchline, 1998). In fact, copepods are characterized by paddle-shaped appendages that function as filters for collecting food from the environment, even though many species are also raptorial. Copepods, with more than 11,500 species (Humes, 1994), show a variety of shapes and sizes (Figure 1.2). The smallest adults measure <0.1 mm (e.g. *Sphaeronellopsis monothrix*, a parasite of ostracods) and the largest measure up to 23 cm (*Penella balaenopterae*, whale parasites).

Copepods act as grazers of photoautotrophs and microheterotrophs, as prey for diverse invertebrates and vertebrates sustaining important fisheries, as extremely sensitive indicators of environmental change and as carriers of carbon between trophic levels. Some species are also parasites of economically important fish species or vectors of human disease (e.g. cholera).

The biology of copepods is rather well known (Mauchline, 1998) with several studies addressing mechanisms of diapause, developmental biology, and host-pathogen interactions. Despite their central importance in sustaining the carbon cycle and important fisheries, there are currently few publically available sequences and this lack of available genomic resources for copepods has proven a major barrier to the wider application of molecular methods in copepod research.

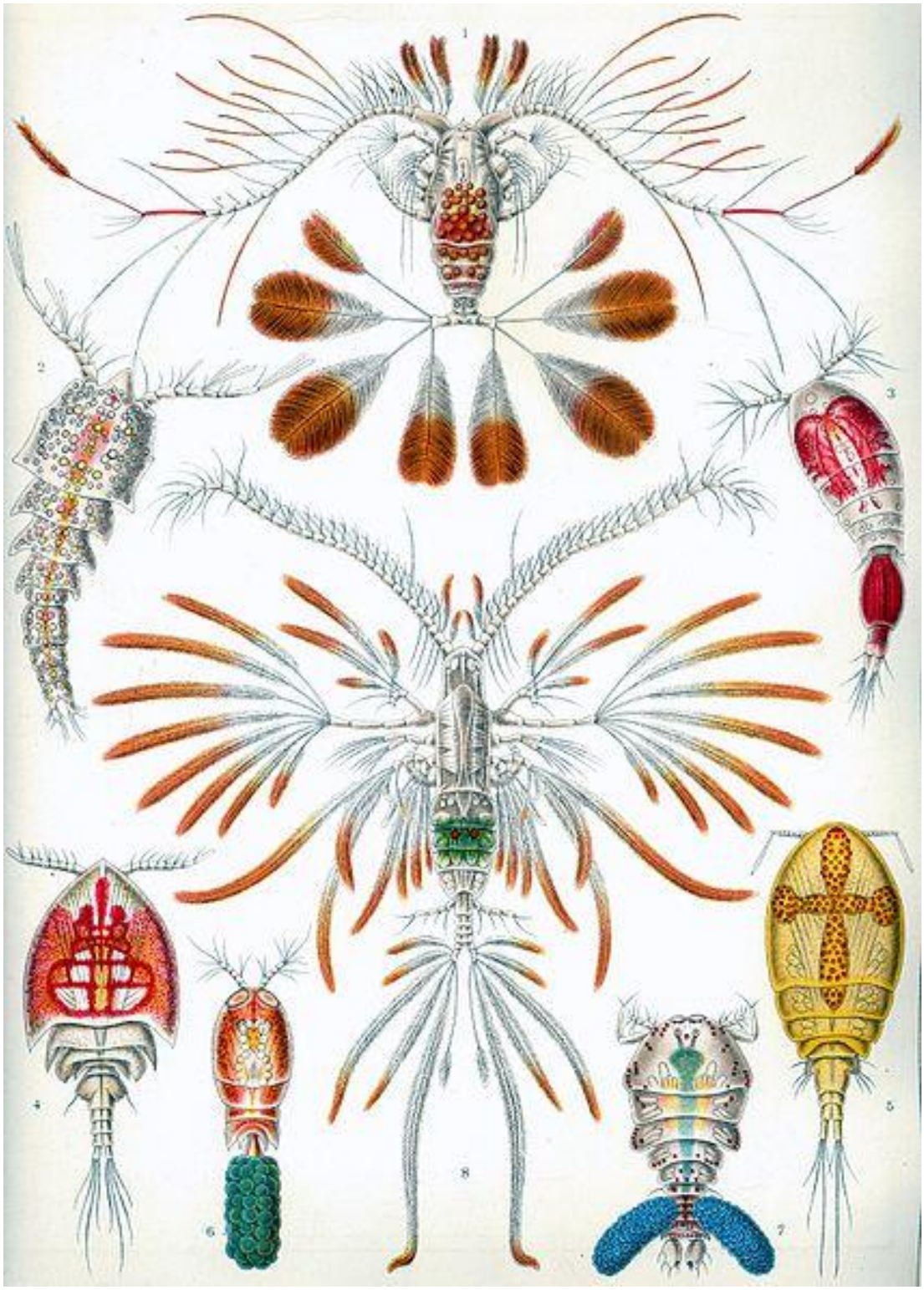


Figure 1.2. Copepods from Ernst Haeckel's Kunstformen der Natur.

Few studies report gene expression patterns induced in copepods exposed to physical (temperature and salinity) and chemical (heavy metals, endocrine disruptor chemicals, H_2O_2, hydrocarbons, diatom toxins, and other toxicants) stressors. A recent review on copepod stress response to these stressors has just been published by Lauritano et al. (Lauritano, *et al.*, 2011c) (Figure 1.3).

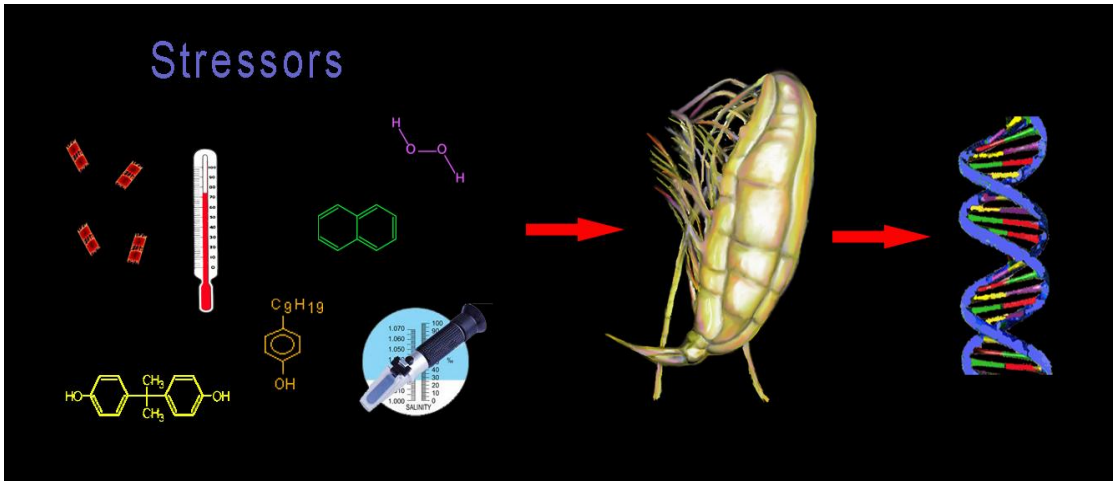


Figure 1.3. This graphical abstract (Lauritano et al., 2011c) shows some of the stressors tested on copepods to evaluate expression level changes of specific genes.

This paper reviews the literature on the defense systems, including detoxification enzymes and proteins (e.g. glutathione S-transferases, heat shock proteins, superoxide dismutase and catalase), studied in copepods at the molecular level including some of the results reported in this thesis. The data indicate high inter- and intra-species variability in copepod response, depending on the type of stressor tested, the concentration and exposure time, and the enzyme isoform studied.

Members of the genus *Calanus* are among the largest copepods and are of great importance in the diet of the juvenile stages of some economically important fish (such as cod, haddock, herring and mackerel) (Gaard, Reinert, 2002; Gislason, Astthorsson,

2002; Ringuette, *et al.*, 2002). The model species I have worked on during the course of my PhD thesis is the copepod *Calanus helgolandicus* (*C. helgolandicus*) (Figure 1.4, 1.5) which is a widespread epipelagic copepod species whose geographical range extends from the temperate Atlantic Ocean to the northern Mediterranean Sea (Bonnet, *et al.*, 2005).

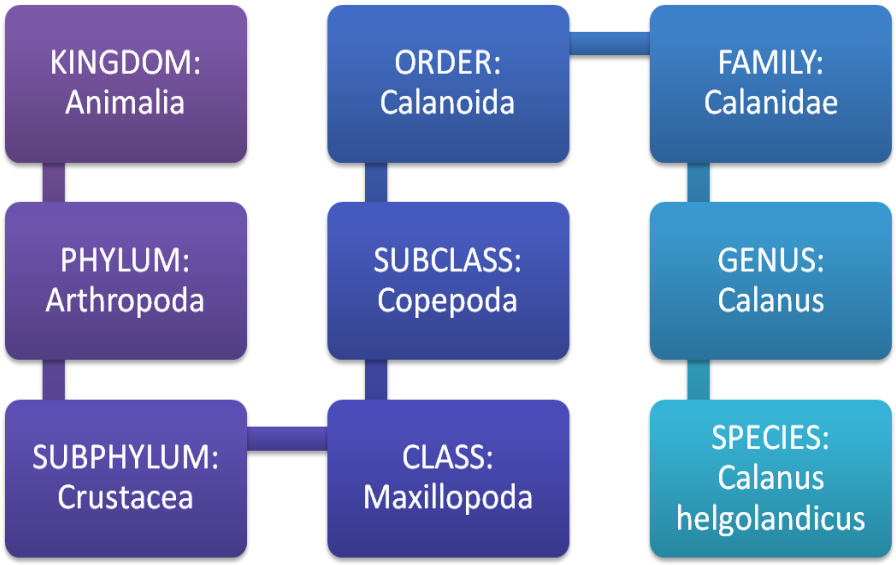


Figure 1.4. *Calanus helgolandicus* scientific classification.

At a European scale, especially in the North Sea, *C. helgolandicus* has progressively become more abundant and widely distributed, while its congeneric species *Calanus finmarchicus*, adapted to live in colder waters, has migrated northwards. These shifts in distribution provide a strong stimulus for further studies on *C. helgolandicus*. Moreover, if climate warming continues, *C. helgolandicus* will probably expand geographically. *C. finmarchicus/helgolandicus* ratio is currently being used by the European Environmental Agency as a climate change impact indicator (Bonnet, *et al.*, 2005). *C. helgolandicus* typically lives in warmer waters, between 5 and

28°C, and with a salinity range between 32-39 ppt (Bonnet, *et al.*, 2005; Unal, *et al.*, 2006).

Figure 1.5. *Calanus helgolandicus* adult female.

In the Mediterranean Sea, *C. helgolandicus* commonly occurs in the North Adriatic Sea where it represents the main diet of the juvenile stages of some economically important fish species and plays a key role in the food web affecting the biological productivity in this marine ecosystem. In addition, it occurs in concomitance with a winter phytoplankton bloom which represents a fundamental food source for copepods. Induced chemical defenses in response to grazing have been shown for several bloom-forming algae (i.e. diatoms and dinoflagellates). Some of these produce

defensive metabolites that have been reported to have multiple simultaneous functions including antipredator, allelopathy, antibacterial, and cell to cell signaling (e.g. diatom polyunsaturated aldehydes PUAs). In the successive two sections I will briefly introduce two important phytoplankton groups: diatoms and dinoflagellates.

1.3 Diatoms

Diatoms are eukaryotic unicellular plants that constitute one of the major components of marine phytoplankton, comprising up to 40% of annual productivity at sea (Falkowski, 1994) and representing 25% of global carbon-fixation (Nelson, *et al.*, 1995). Copepods feed willingly on diatoms and have evolved strong teeth-like structures to break the strong silica wall of diatom cells. There are more than 200 genera of living diatoms, with about 100.000 species (Figure 1.6).

Diatoms have traditionally been considered a prefered food for zooplankton grazers such as copepods and for the transfer of organic carbon to higher trophic levels sustaining important fisheries (Mauchline, 1998) (Figure 1.1).

However, numerous studies have shown that these unicellular plants at times produce secondary metabolites with toxic effects on reproductive processes in crustacean copepods (Fontana, *et al.*, 2007b; Ianora, *et al.*, 2004; Miralto, *et al.*, 1999) and cladocerans (Carotenuto, *et al.*, 2005), echinoderm sea urchins (Romano, *et al.*, 2010) and sea stars (Caldwell, 2009; Guenther, *et al.*, 2009), polychaete worms (Caldwell, *et al.*, 2002; Simon, *et al.*, 2010), and ascidians (Tosti, *et al.*, 2003). Diatom metabolites, collectively termed oxylipins, are the end-products of a lipoxygenase/hydroperoxide lyase metabolic pathway (Cutignano, *et al.*, 2006;

d'Ippolito, *et al.*, 2004; d'Ippolito, *et al.*, 2009; Fontana, *et al.*, 2007a; Pohnert, 2000) initiated by damage to algal cells, as occurs through grazing by predators. Cell damage activates lipase enzymes, which liberate polyunsaturated fatty acids (PUFAs) from cell membranes that are immediately oxidized and cleaved within seconds to form polyunsaturated aldehydes (PUAs) and a plethora of other metabolites collectively termed oxylipins (Figure 1.7).

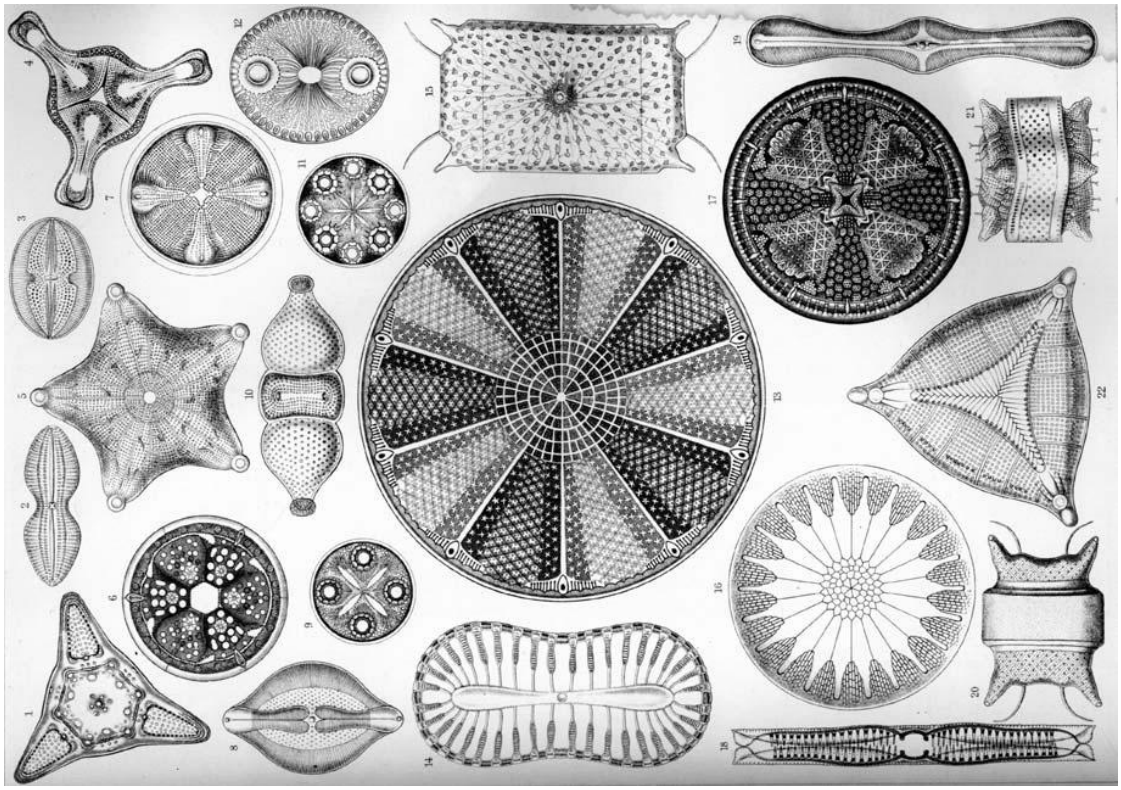


Figura 1.6. Diatoms from Ernst Haeckel's "Kunstformen der Natur" (1904).

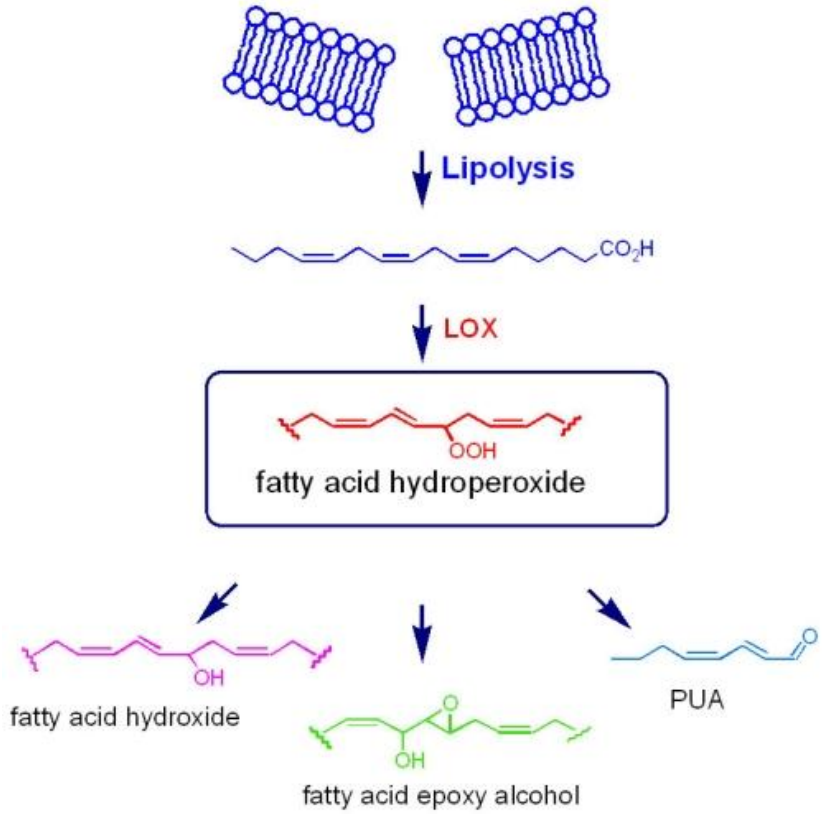


Figure 1.7. Biosynthetic sketch for the synthesis of oxylipins in marine diatoms (Fontana et al., 2007).

Oxylipins, and PUAs in particular, have important biological and biochemical properties including the disruption of gametogenesis, gamete functionality, fertilization, embryonic mitosis, and larval fitness and competence (Caldwell, 2009). Although the effects of such toxins are less catastrophic than those inducing poisoning and death of predators, they are none-the-less insidious inducing abortions, birth defects and reduced larval survivorship (Ianora, *et al.*, 2004; Miralto, *et al.*, 1999) (Figure 1.8).

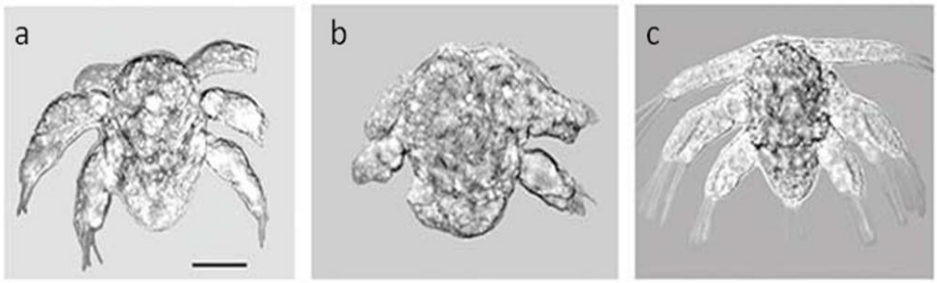


Figure 1.8. Effects of diet on *C. helgolandicus* offspring fitness. a, b, After feeding on *Skeletonema marinoi*, such nauplii were strongly deformed. c, Nauplii generated from females fed the control diet *Prorocentrum minimum* appeared normal. Scale bar, 90 μm (from Ianora et al., 2004).

Such antiproliferative compounds may discourage herbivory by sabotaging future generations of grazers, thereby allowing diatom blooms to persist when grazing pressure would otherwise have caused them to crash.

The specific type and quantity of oxylipins produced differs between diatom species and strains due to a variety of precursor PUFAs and enzymes with variable effects on grazers (Ianora, Miralto, 2010). Oxylipin production also differs depending on the physiological status of the same diatom culture due to e. g. different nutrient regimes (Ribalet, *et al.*, 2007; Ribalet, *et al.*, 2009) or various growth stages (d'Ippolito, *et al.*, 2009; Ribalet, *et al.*, 2007; Vidoudez, Pohnert, 2008). Similar wound-activated compounds are also found in terrestrial plants where they play a pivotal role in defense because of their antibacterial, wound healing and antiproliferative activity (Andreou, *et al.*, 2009).

1.4 Dinoflagellates

Dinoflagellates are unicellular protists which exhibit a great diversity of forms (Figure 1.9). Their name derives from the greek dinos "whirling" and latin flagellum "whip, scourge".

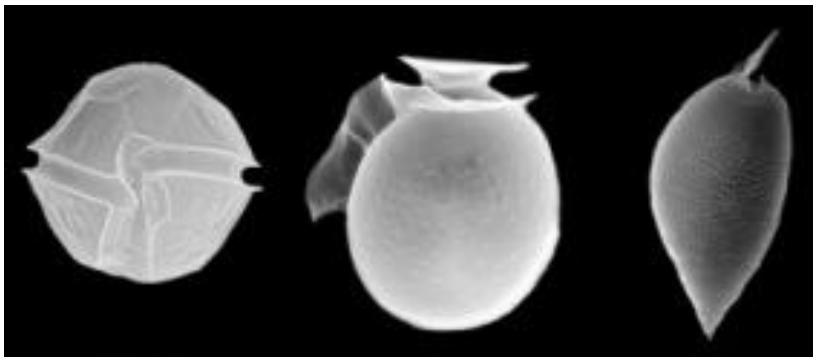


Figure 1.9. Three dinoflagellate structures from Stazione Zoologica Anton Dohrn website (www.szn.it).

Many are photosynthetic, manufacturing their own food using the energy from sunlight, some species are capable of producing their own light through bioluminescence, while other dinoflagellates are parasites on fish or on other protists. Dinoflagellates are the largest group of marine eukaryotes aside from the diatoms and primary producers making them an important part of the aquatic food web (Figure 1.1). Most are marine but they are also common in fresh water habitats as well.

Many phytoplankton species are known to form harmful algal blooms (HABs), including: dinoflagellates, diatoms, haptophytes and raphidophytes. The species within these different groups show variable abundance/biomass and toxicity patterns. The most dramatic effect of some species (e.g. the dinoflagellate *Karenia brevis*, figure 1.10) is to

cause "blooms" in such great numbers that the water may appear golden or red, producing a "red tide" (Figure 1.11) (Landsberg, 2002; Van Dolah, *et al.*, 2009).

Many algae produce secondary metabolites which can adversely affect other organisms. In general, the major producers of toxins in the marine environment are the dinoflagellates and diatoms. Dinoflagellates can produce neurotoxins (e.g. brevetoxin A, the most potent neurotoxin secreted by the dinoflagellate *Karenia brevis*) which affect muscle function in susceptible organisms (Landsberg, 2002; Nicolaou, *et al.*, 1998). Humans may also be affected by eating fish or shellfish containing the toxins. Gastrointestinal, neurosensory, neurocerebellar, neuromuscular, systemic and general CNS effects are the common deseases induced by dinoflagellate toxins, also known as neurotoxic shellfish poisoning (NSP) (Watkins, *et al.*, 2008).

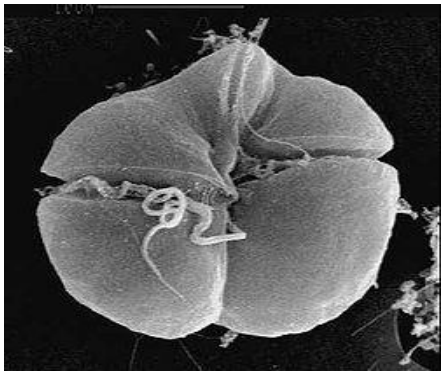


Figure 1.10. Scanning electron micrograph of *Karenia brevis*. Micrograph: Florida Marine Research Institute (http://www.bioone.org/doi/abs/10.1641/0006-3568%282003%29053%5B0918%3AHABBPN%5D2.0.CO%3B2).

Figure 1.11. Red algal bloom at Leigh, near Cape Rodney. Photo by Miriam Godfrey (from http://serc.carleton.edu/microbelife/topics/redtide/general.html).

1.5 Thesis aim

The object of this thesis was to investigate the defense systems and stress reponses in adult copepods exposed to toxic diatom and dinoflagellate diets using a molecular approach.

mRNA expression patterns induced by feeding on diatoms or dinoflagellates were examined by using Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR). mRNA expression changes are an important early signal compared to other physiological parameters and/or phenotype changes.

1. The first part of the thesis was a set up for gene expression studies in this copepod species. In order to have enough animals for the molecular experiments, a cultivating breeding system was set up with researchers at the Stazione Zoologica Anton Dohrn where I conducted my PhD work (Carotenuto, Esposito, Pisano, Lauritano *et al*., 2011, submitted). Then I selected and screened a list of putative reference genes (RGs), necessary for the RT-qPCR analyses, by using three different softwares (BestKeeper, NormFinder and GeNorm). Once the best RGs were obtained, I analyzed, for the first time, the effects of the ingestion of the toxic oxylipin-producing diatom *Skeletonema marinoi* by the calanoid copepod *Calanus helgolandicus* by evaluating expression levels of specific genes of interest, the microtubule subunits alpha and beta tubulins, after 2 days of feeding (Lauritano *et al*., 2011a).
2. To further investigate gene expression patterns induced by *S. marinoi* on defence systems in *C. helgolandicus*, the second part of the thesis focused on analyzing a

series of genes involved in generic stress response, aldehyde detoxification and apoptosis regulation in this copepod species (Lauritano *et al*., 2011b).

3. A third part of the thesis was concentrated on analyzing gene expression changes with time-series experiments for two different *C. helgolandicus* populations (North Adriatic Sea and North Atlantic Ocean) fed the same toxic diatom diet (Lauritano et al., in preparation). The experiments were in part carried out at the Station Biologique de Roscoff and were financed by the EU FP7 ASSEMBLE project. The data provided interesting results on common or population-specific responses in expression patterns in copepods.
4. Then I examined the effect of a *Skeletonema marinoi*-dominated spring bloom in the Northern Adriatic Sea in 2010 and 2011. Field zooplankton samples were also collected during the oceanographic cruise ENVADRI (Lauritano *et al*., in preparation)(Bastianini, *et al.,* 2011).
5. Finally, I analysed gene expression changes when *C. helgolandicus* was exposed to the toxic dinoflagellate *Karenia brevis* compared to when *C. helgolandicus* was exposed to toxic diatom diets (Lauritano *et al*., in preparation).

In order to better examine each topic, I will separately present and discuss each one in separate chapters reporting a brief specific introduction, methods, results and discussion. Finally, I will summarize and conclude my thesis.

Chapter 2:

First molecular evidence of diatom effects in the copepod *Calanus helgolandicus* on microtubule subunits

Published as:

Lauritano Chiara, Borra Marco, Carotenuto Ylenia, Biffali Elio, Miralto Antonio, Procaccini Gabriele and Ianora Adrianna. (2011)
First molecular evidence of diatom effects in the copepod *Calanus helgolandicus.* Journal of Experimental Marine Biology and Ecology, 404, 79-86

2.1. Brief introduction

Buttino and co-workers (Buttino, *et al.*, 1999) were the first to show that water-soluble extracts of the diatom *Thalassiosira rotula* induced aberrations in embryonic tubulin organization leading to cell blockage and the absence of spindle formation in the sea urchin *Paracentrotus lividus*, but the molecules responsible for these effects were unknown at the time. Miralto *et al*. (Miralto, *et al.*, 1999) later isolated the PUAs 2-trans-4-cis-7-cis-decatrienal, 2-trans-4-trans-7-cis-decatrienal and 2-trans-4-trans-decadienal and showed that they arrested embryonic development of copepod and sea urchin embryos in a dose-dependent manner, and also had antiproliferative and apoptotic effects on human carcinoma cells. Hansen *et al*. (Hansen, *et al.*, 2004) studied the effects of decadienal on the sea urchin *Sphaerechinus granularis* and showed that this PUA inhibited cyclin B/Cdk1 kinase activity and DNA replication. Staining of alpha-tubulin subunits showed that tubulin polymerization was disrupted and aberrations were induced in mitotic spindles (Hansen, *et al.*, 2004).

Tubulins are proteins that are the building blocks of microtubules (MTs), one of the active components of the cytoskeleton. MTs play an important role in many cellular functions including development and maintenance of cell shape, growth, signalling, protein movement, intracellular vesicle transport, organization and positioning of membranous organelles, and segregating replicated chromosomes into daughter cells during mitosis and meiosis (Calligaris, *et al.*, 2010; Harrison, *et al.*, 2009; Jordan Mary Ann, 2004; Nogales, *et al.*, 1998).

MTs consist in α- and β-tubulin monomers which constantly switch between a state of polymerization and depolymerization (Calligaris, *et al.*, 2010; Nogales, *et al.*,

1998), the ratio of which can be altered by many natural products and drugs, especially anti-cancer compounds targeting MTs (Calligaris, *et al.*, 2010; Harrison, *et al.*, 2009; Jordan Mary Ann, 2004; Sashidhara, *et al.*, 2009). Compounds that bind to tubulin modify the formation and function of MTs and can affect the proper functioning and formation of the mitotic spindle. If MT function is altered and spindle dynamics is compromised, a mitotic block or the slowing down in cell cycle progression occurs at the metaphase-anaphase transition, eventually leading to apoptosis (Jordan Mary Ann, 2004).

The aim of this chapter was to evaluate, for the first time, the effects of ingestion of the oxylipin-producing diatom *Skeletonema marinoi* (SKE) by the copepod *Calanus helgolandicus* on α- and β-tubulin gene expression levels using the reverse transcription-quantitative real time polymerase chain reaction (RT-qPCR). Previous studies have already shown that a diet of *S. marinoi* which contains high levels of PUAs and other oxylipins (same strain as in this study) reduces egg hatching success and female survival in this copepod species (Fontana, *et al.*, 2007b; Ianora, *et al.*, 2004), with a concomitant appearance of apoptosis in both copepod embryos and female tissues (Buttino, *et al.*, 2008), but there are no studies on gene expression analyses in the copepod *C. helgolandicus*. We also investigated tubulin gene expression levels in *C. helgolandicus* feeding on the non-oxylipin producing flagellate *Rhodomonas baltica* (RHO) and dinoflagellate *Prorocentrum minimum* (PRO) currently being used to rear *C. helgolandicus* in our laboratory. *P. minimum* was considered the control diet since several previous studies have shown that *C. helgolandicus* reproduces and grows well on this diet (Ianora, *et al.*, 2004). Although the biology of *C. helgolandicus* species is rather well known (Mauchline, 1998), very little is known about its genome, except for the gene sequences of cytochrome oxidase subunit I, antennapedia proteins 1 and 2,

cytochrome b, and 16S, 18S and 28S ribosomal RNA as reported in the public database PubMed. Since there are few studies on gene expression analysis via RT-qPCR in copepods (Frost, Nilsen, 2003; Hansen, *et al.*, 2008a; Hansen, *et al.*, 2008b; Hansen, *et al.*, 2009; Hansen, *et al.*, 2011; Kvamme, *et al.*, 2004; Lee, *et al.*, 2008; Lee, *et al.*, 2007; Seo, *et al.*, 2006a; Skern-Mauritzen, *et al.*, 2009; Tarrant, *et al.*, 2008), and none of these focus on *C. helgolandicus*, here we also describe the first evaluation of reference genes as internal controls for RT-qPCR analyses in *C. helgolandicus*. It is important to note that the analyses of potential reference genes were conducted according to the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) suggestions and checklist (Bustin, *et al.*, 2009; Bustin, *et al.*, 2010).

2.2 Methods

2.2.1 Collection of copepods

Calanus helgolandicus specimens were collected in the North Adriatic Sea from March to April 2009 and transported to Naples where they were placed in a 500 L re-circulating copepod breeding system (Buttino, *et al.*, 2011) maintained at 20°C, 38 ppm salinity, 12 h:12 h light:dark cycle, and a mixed diet of the flagellates *Rhodomonas baltica*, *Prorocentrum minimum* and *Isochrysis galbana*.

Adult female *C. helgolandicus* specimens were sampled from the culturing tanks, isolated under a Leica stereomicroscope and transferred to 10 L beakers filled with 0.22 μm filtered sea water at 20°C. Beakers containing from 5 to 40 adult females were fed either unialgal diets of the control non-oxylipin producing dinoflagellate *P. minimum* (PRO) (6000-8000 cells/ml), or test diets of the oxylipin-producing diatom

Skeletonema marinoi (SKE) (45.000-60.000 cells/ml) and the non-oxylipin producing flagellate *R. baltica* (RHO) (7500-8000 cells/ml). The strains belong to the Stazione Zoologica Anton Dohrn culture collection. *R. baltica* and *P. minimum* were cultured in 2-l glass jars with 0.22 µm-filtered FSW enriched with *k* medium at 20^{o}C and on a 12:12 hr dark:light cycle. The diatom *S. marinoi* was cultured under the same experimental conditions but with F2 medium.

Copepods were collected from the culturing tank on three different occasions (Table 2.1). The first sampling was conducted in May 2009 when two replicates of 20 adult *C. helgolandicus* were fed PRO (replicates designated as PRO1M and PRO2M, respectively) and another two were fed SKE (replicates designated as SKE1M and SKE2M, respectively). The second collection was in July 2009 when four groups of 5 animals were fed PRO (replicates designated as PRO1L, PRO2L, PRO3L and PRO4L, respectively) and another four were fed SKE (replicates designated as SKE1L, SKE2L, SKE3L and SKE4L, respectively). The third sampling was in September when a third algae was introduced, RHO. Three groups of 40 specimens were fed RHO (replicates designated as RHO1S, RHO2S and RHO3S, respectively) and three were fed SKE (replicates designated as SKE1S, SKE2S and SKE3S, respectively).

During each sampling, animals were fed for two days *ad libitum* on either one of the three algal diets and were then transferred to filtered sea water (FSW) for 24h to eliminate any algal residues in the gut. After this, each replicate was washed in 50 µl of FSW and carefully transferred to 500 µl Trizol Reagent (Invitrogen), frozen directly in liquid nitrogen and stored at -80°C for few weeks until RNA extraction. To study the extent to which the selected genes were differentially expressed in our experimental conditions, we extracted RNA and retro-transcribed it in Complementary DNA (cDNA)

(double-stranded DNA version of an mRNA molecule), which was used as the template for our molecular analyses.

SAMPLING TIME	SPECIMEN NUMBER	FEEDING	ABBREVIATION
MAY	20	PRO	PRO1M
	20	PRO	PRO2M
	20	SKE	SKE1M
	20	SKE	SKE2M
JULY	5	PRO	PRO1L
	5	PRO	PRO2L
	5	PRO	PRO3L
	5	PRO	PRO4L
	5	SKE	SKE1L
	5	SKE	SKE2L
	5	SKE	SKE3L
	5	SKE	SKE4L
SEPTEMBER	40	RHO	RHO1S
	40	RHO	RHO2S
	40	RHO	RHO3S
	40	SKE	SKE1S
	40	SKE	SKE2S
	40	SKE	SKE3S

Table 2.1. Copepod collections including information on sampling month, number of specimens fed and frozen for RNA extraction, algae used for feeding experiments and abbreviation of the treated groups.

2.2.2 RNA extraction and quantification

Total RNA was extracted using a modified Trizol manufacturer's protocol (Invitrogen) (For more details see Appendix 1). To remove hypothetically contaminating DNA, each sample was treated with DNaseI (Invitrogen) according to the instruction manual. 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. All samples were free from protein and organic solvents used during RNA extraction. RNA quality was also evaluated by gel electrophoresis showing minimal degradation of RNA, which was almost completely intact, with sharp 18S and 28S ribosomal bands.

2.2.3 cDNA synthesis

The amount of RNA used for the reverse transcription steps was always 1 μg. This amount of RNA was converted into cDNA with the ProScript First Strand cDNA Synthesis Kit (New England Biolabs), following the manifacturer's instructions, and using the *GeneAmp PCR System 9700* (Perkin Elmer).The first reaction was carried out in 16 μl final volume with 1 μg of RNA, 50μM of dt_{23} VN Primer, 15 μM of Random Primer 9, 2.5 μM of dNTP Mix and H_2O. The mix was first denatured by heating at 70°C for 5 min. A second mix with 1% RT buffer, 10 units/μl of RNase Inhibitor and 25 units/μl of M-MuLV was added to reach a final volume of 20 μl, and the total reaction mix was incubated at 42°C for 90 min and at 95°C for 5 min. RNase H (2units/μl) was added to eliminate non-converted (or potential contaminant) RNA by incubating at 37°C for 20 min, followed by 5 min at 95°C to stop the reaction. To

evaluate the efficiency of cDNA synthesis, a PCR was carried out with primers of a constitutive gene, S20. The reaction was carried out on the *GeneAmp PCR System 9700* (Perkin Elmer) in 20 µl final volume with 2 µl 10× PCR reaction buffer Roche, 2 µl 0.1% BSA, 2 µl 10× 2mM dNTP, 0.8 µl 5U/µl Taq Roche, 1 µl 20 pmol/µl of each oligo, 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 sec, 60°C for 1 min and 72°C for 30 sec, and a final extension step at 72°C for 7 min.

2.2.4 Primer design

To perform RT-qPCR in this copepod species we designed specific primers to isolate and amplify selected genes. Primers for hypothetical Reference Genes (RGs) and Genes of Interest (GOI) were designed considering the alignment of conserved domains in different arthropod species such as *Calanus finmarchicus*, *Calanus californicus*, *Tigriopus japonicus*, *Homarus americanus, Drosophila melanogaster* and *Anopheles gambiae*. Alignments were performed with Clustal W (Clustal) and BioEdit (BioEdit). Gene Runner, V3.05 (Hasting Software) was used to predict primer melting temperature (T_m) and check if primers formed dimers, hairpin, bulge and internal loops. The primers for 18S were designed from the known sequence (Accession Number: **AY446908**) using Primer3 software, v. 0.4.0. RGs belonging to different functional classes were selected in order to reduce the possibility that they might be co-regulated (e.g. S20, S7 and S18). The selected RGs were: β-actin (ACT), elongation factor 1α (EFA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal units (18S, S7, S20), adenosine-3-phosphate synthase (ATPs), histone 3 (HIST). The GOI were the two microtubule subunits: α-tubulin (α tub) and β-tubulin (β tub). Table 2.2 lists gene functions, primers' sequences, amplicon size, correlation coefficient (R^2) and efficiency

(E^a). Primers were synthesized commercially by Primm Labs. Primers were designed to amplify cDNA regions ranging from 100 to 200 bp in size, in order to facilitate cross-comparison of assays and assure equal PCR efficiencies. PCR conditions were optimized on 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× 2mM dNTP, 0.8 μl of 5U/μl Taq Roche, 1 μl of 20 pmol/μl for each oligo, 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 sec, 60°C for 1 min and 72°C for 30 sec, and a final extension step at 72°C for 7 min. Amplified PCR products were analyzed by 1.5% agarose gel electrophoresis in 1X 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 to the *QIAquick Gel Extraction Kit* protocol (QIAGEN) and sequence 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 analysed on the Automated Capillary Electrophoresis Sequencer *3730 DNA Analyzer* (Applied Biosystems). The identity of each sequence was confirmed using the bioinformatics tool BLAST (Basic local alignment search tool) (BLAST). The sequences are deposited in GenBank under the Accession Numbers shown in Table 2.2.

Gene name	Acc. no.	Function	Primer sequence 5'-3' (forward and reverse)	Amplicon size	E^a	R^2
Reference genes						
EFA	HQ270534	Translational elongation factor	GACAAGCCCCTCAGACTTCC GGAGAGACTCGTGGTGCATC	172	97%	0.9998
ATPs	HQ270507	Synthesis ATP	CTCCATCACTGACGGACAGATC TCAAGCTTCATGGAACCAGC	150	100%	0.9986
Hist3	HQ270530	Structure of chromatin	GAGGAGTGAAGAAGCCCCAC TGAAGTCCTGAGCAATCTCCC	137	100%	0.9969
18S	AY446908	Ribosomal protein	GAAACCAAAGCATTTGGGTTC GCTATCAATCTGTCAATCCTTCC	164	89%	0.9958
GAPDH	HQ270535	Oxidoreductase in glycolysis and gluconeogenesis	ATCTTTGATGCCAAGGCTGG GTCCTTGCCCTGCATGAAG	126	91%	0.9704
S20	HQ270531	Ribosomal protein	CGTAAGACTCCTTGTGGTGAGG GAAGTGATCTGCTTCACGATCTC	113	89%	0.9915
S7	HQ270532	Ribosomal protein	CGTGAGCTGGAAAAGAAGTTC CAGGATGGAGTTGTGGACAG	149	100%	0.9934
Ubi	HQ270536	Proteins degradation	GCAAGACCATCACCCTTGAG CAGCGAAAGATCAACCTCTG	113	100%	0.9984
Actin	HQ270533	Cytoskeleton structure	GGCACCACACTTTCTACAACG GTTGAAGGTCTCGAACATGATC	131	93%	0.9993
Genes of interest						
α tub	HQ270529	Microtubule subunit	ACAGCTTCTCCACCTTCTTCTC GTTGTTGGCGGCATCCTC	168	94%	0.9997
β tub	HQ270528	Microtubule subunit	GGATTTCAGCTGACCCACTC GTCTCATCAGTATTTTCCACCAG	206	97%	0.9862

[a] = Oligo efficiency

Table 2.2. PubMed accession numbers for candidate reference genes (RG) and genes of interest (GOI) for *Calanus helgolandicus* qPCR assays. Putative RGs were elongation factor 1α (EFA), adenosine 3-phosphate synthase (ATPs), histone 3 (HIST), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal units (18S, S7, S20), ubiquitin (UBI), β-actin (ACT) and GOIs were α- and β- tubulins. Gene functions, primer sequences, amplicon sizes, oligo efficiencies (E^a) and correlation factors (R^2) are also given.

2.2.5 Reverse transcription-Quantitative real time polymerase chain reaction (RT-qPCR)

The fluorescent dye SYBR GREEN was used to value expression levels of the selected genes by RT-qPCR. This dye shows maximal fluorescence only with double-strand DNA and was used to detect our amplicons (For details see APPENDIX 2).

Three different algorithms were utilized to identify the best reference genes in our experimental design: BestKeeper (Pfaffl, *et al.*, 2004), geNorm (Vandesompele, *et al.*, 2002) and NormFinder (Andersen, *et al.*, 2004).

To study the expression of each target gene relative to the most stable RGs, we used REST tool (Relative expression software tool) (Pfaffl, *et al.*, 2002). This tool used a mathematical model based on the PCR efficiencies and the mean crossing point deviation between the sample and the control group. The advantage of REST is that this software tool tests the group differences for significance with the Pair-Wise Fixed Reallocation Randomization Test (Pfaffl, *et al.*, 2002).

Statistical analysis was performed using GraphPad Prim statistic software, V4.00 (GraphPad Software).

2.3 Results

2.3.1 Validation of best reference genes for RT-qPCR

Raw Ct data of potential RGs are reported in Figure 2.1. According to the mathematical approach of BestKeeper, RG expression stability considers the standard deviation of the Ct values (Table 2.3). Hence the most stable RGs have a standard deviation (SD) lower than 1 and these were S20, S7, GAPDH, HIST and UBI.

BestKeeper analysis (Pfaffl, *et al.*, 2004) indicated S20 as the most stable gene, followed by S7 and GAPDH.

GeNorm analysis (Vandesompele, *et al.*, 2002) confirmed the results of BestKeeper, showing that the two most stable genes, with the lowest expression stability (M), were S20 and S7 (Figure 2.2). Pair-wise variation was subsequently calculated to evaluate the effect of adding another RG to those already analyzed. Below the cut-off value of 0.15 the inclusion of additional RGs was not required. According to our results (Figure 2.3), the two best RGs, S20 and S7, were sufficient for the analysis.

According to the statistical approach of NormFinder, our best candidate reference genes, with the lowest stability values, were S20, S7 and GAPDH, as reported in figure 2.4. The rank pattern was the same as for Bestkeeper and geNorm analyses as summarized in table 2.4.

Although the three approaches agreed that the best RGs were S20 and S7, we decided to also use the third best RG, GAPDH, because the two ribosomal proteins S20 and S7, belonging to the same functional class, might be co-regulated. Moreover three RGs are suggested as a minimum significant number for reliable assessment of gene expression using qPCR (Bustin, *et al.*, 2010).

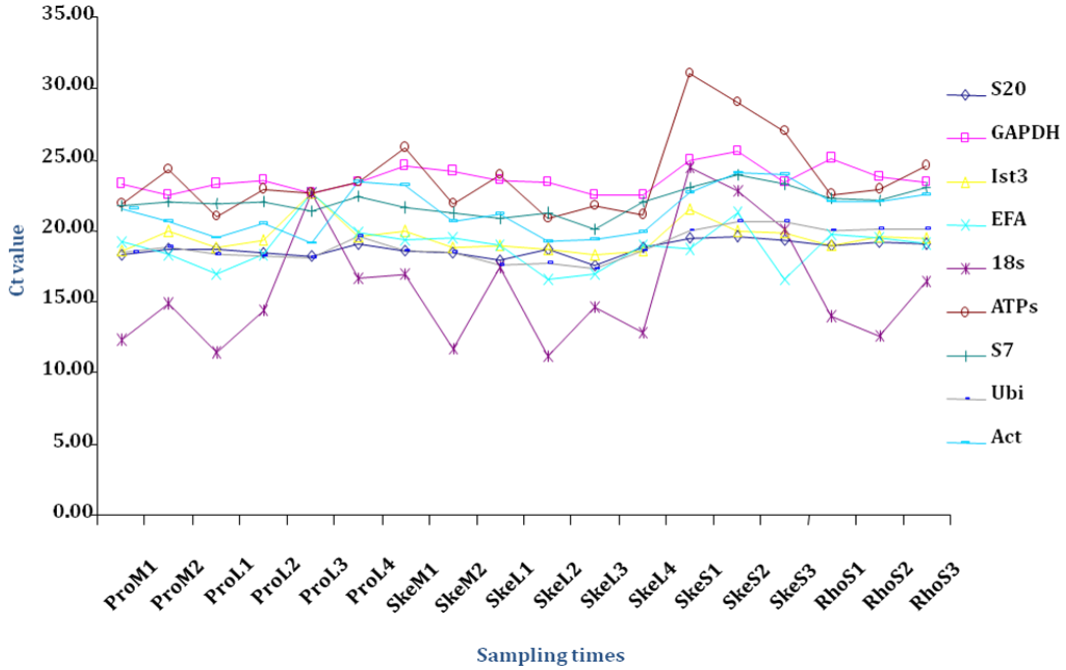


Figure 2.1. Ct value profile.The Ct values (ordinates) obtained for all candidate reference genes (RG) during all samplings (abscissa) for normalization in C. helgolandicus qPCR assays. Each curve represents the degree of stability of Ct values for each RG.

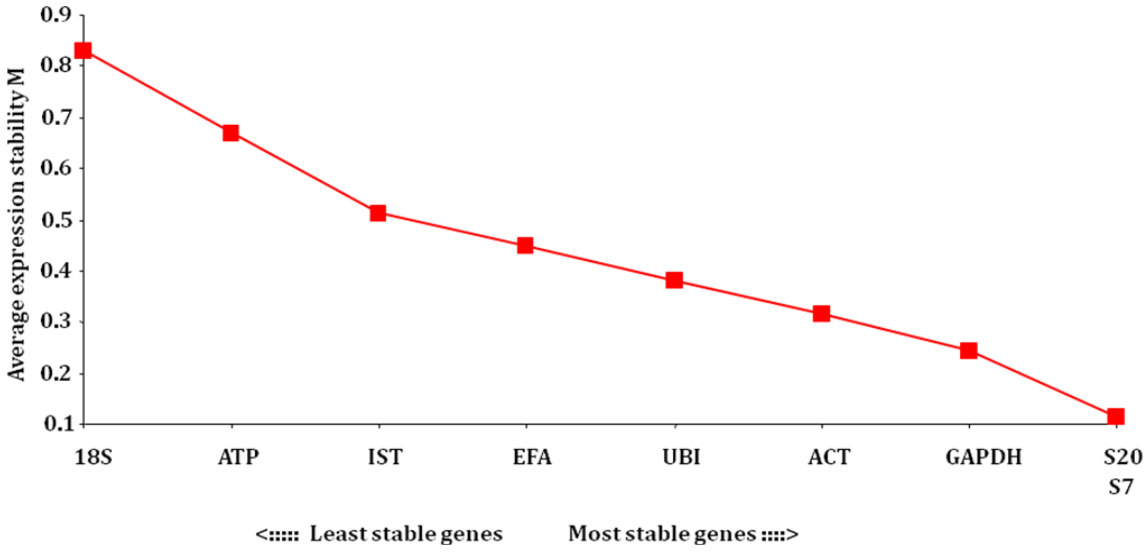


Figure 2.2. Gene expression stability M of candidate RGs calculated by geNorm.

The stepwise exclusion of genes that are more variable among samples using the geNorm program. Data points represent the average expression stability values of remaining RGs.

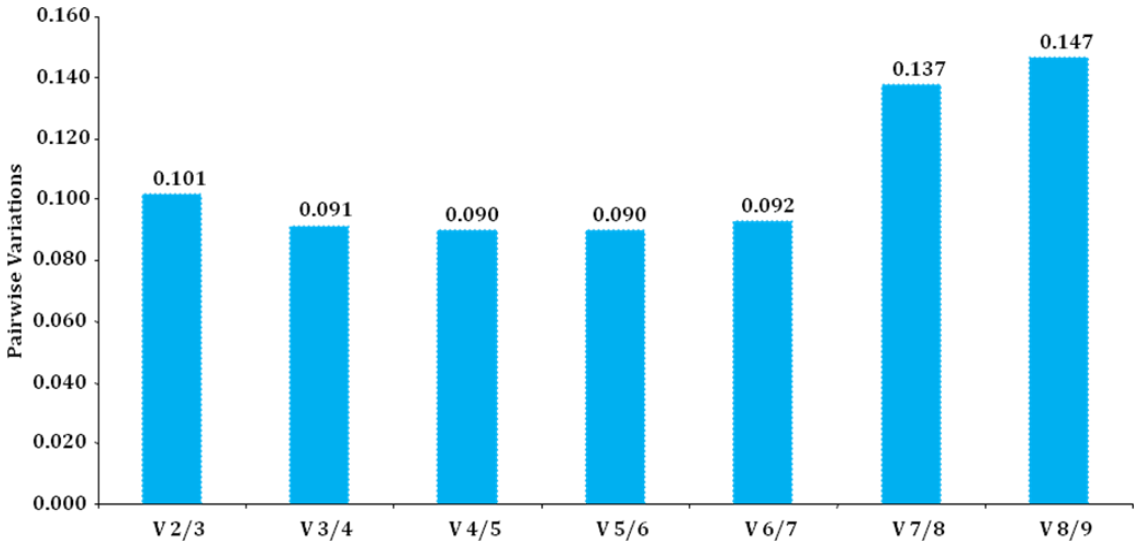


Figure 2.3. Determination of the optimal number of control genes for normalization. Pair-wise variation to evaluate the effect of adding another RG to those already analyzed. The inclusion of additional RGs was not required below the cut-off value of 0.15. The two best RGs, S20 and S7, were sufficient for this analysis, but the third best RG, GAPDH, was also used because the two ribosomal proteins S20 and S7, belonging to the same functional class, might be co-regulated.

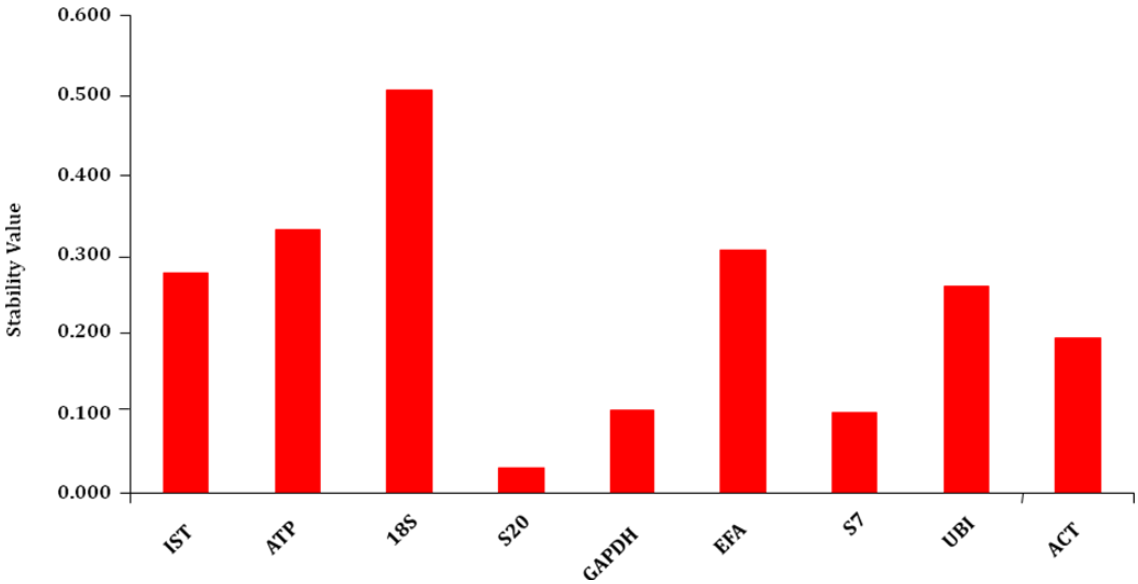


Figure 2.4. Determination of the most stable RG using NormFinder. The NormFinder algorithm ranks the data set of candidate normalization genes according to their expression stability in a given experimental design. Red bars represent the stability values of our candidate genes.

	S20	GAPDH	HIST3	EFA	18S	ATPS	S7	UBI	ACT
n	18	18	18	18	18	18	18	18	18
GM	18.73	23.62	19.52	18.86	15.52	23.66	22	18.91	21.35
AR	18.74	23.64	19.55	18.92	15.98	23.81	22.02	18.94	21.41
min	17.5	22.47	18.29	16.49	11.26	20.83	20.09	17.31	19.15
max	19.65	25.55	22.67	22.67	24.38	31.06	23.9	20.67	24.01
SD	0.42	0.68	0.76	1.11	3.29	2.1	0.67	0.94	1.41
CV	2.22	2.89	3.87	5.85	20.59	8.82	3.05	4.96	6.57

Table 2.3. BestKeeper results. Descriptive statistics of the derived crossing points for each putative RG, where n = number of samples; GM = geometric mean; AR = arithmetic mean; min and max = extreme values of the crossing points (CP); SD = standard deviation of CP; CV = coefficient of variance.

Rank	BestKeeper	GeNorm	NormFinder
1	S20	S20	S20
2	S7	S7	S7
3	GAPDH	GAPDH	GAPDH
4	HIST	ACT	ACT
5	UBI	UBI	UBI
6	EFA	EFA	HIST
7	ACT	HIST	EFA
8	ATPs	ATPs	ATPs
9	18S	18S	18S

Table 2.4. Stability of putative reference genes in Calanus helgolandicus. Candidate RGs are ranked from most to least stable using the three different softwares described in the text. Three best reference genes used for the analysis were S20, S7 and GAPDH.

2.3.2 Expression level of genes of interest (GOI)

To investigate if microtubules in *Calanus helgolandicus* were affected by diets of SKE and RHO, we analyzed the relative expression levels of the two MT subunits: α- and β-tubulin (Figure 2.5). The analyses were carried out on all samples using the three best RGs (S20, S7 and GAPDH) to normalize our data and considering copepods fed PRO as the control group using the Relative expression software tool (REST tool) (Pfaffl, *et al.*, 2002).

Figure 2.5 shows that the expression levels of our GOI in copepods fed on the oxylipin-producing diatom SKE were significantly lower than on the flagellate RHO (students't-test, $p<0.0001$, for both genes). According to REST analysis, α-tubulin was about 2-fold down-regulated while β-tubulin was about 3-fold down-regulated in SKE treated samples. As expected, α- and β-tubulin gene expression in copepods fed RHO compared to copepods fed the control diet PRO did not show significantly different expression levels.

The behaviour of GOI was confirmed by the replicates of the experiments (listed in table1) with a standard deviation (SD) of 0.194 and 0.052 for α-tubulin in SKE and RHO conditions, respectively, and 0.080 and 0.018 for β-tubulin for SKE and RHO, respectively (Figure 2.5).

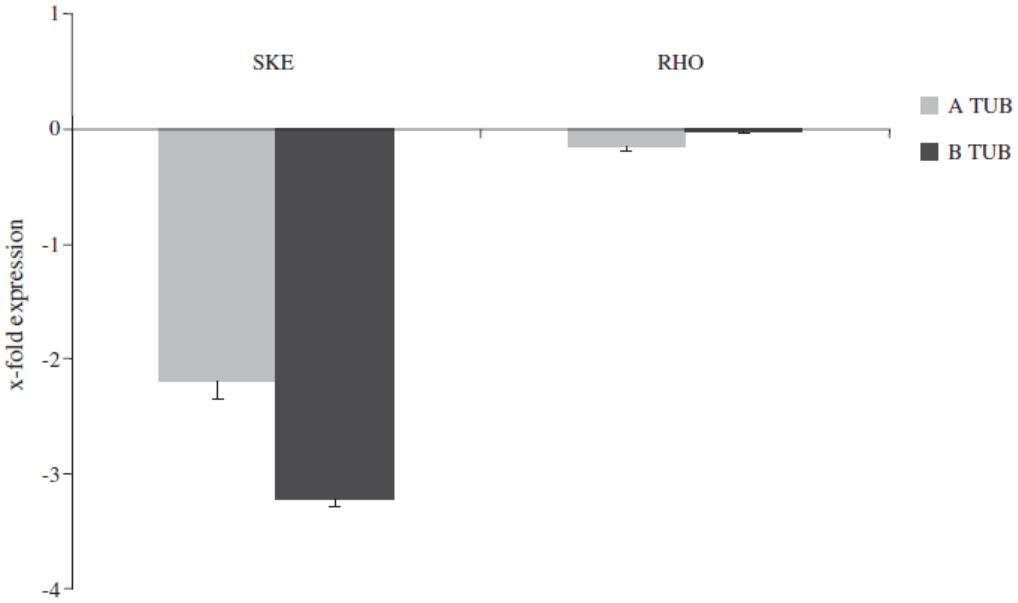


Figure 2.5. Relative target gene expression in copepods fed on *Skeletonema marinoi* and *Rhodomonas baltica*. The figure shows alpha- and beta-tubulin (A TUB and B TUB, respectively) gene expression levels (y-axis, Mean ± SD) in copepods fed on the test diatom *Skeletonema marinoi* (SKE) and the flagellate *Rhodomonas baltica* (RHO), respectively. The data are normalized with our three best reference genes, S20, S7 and GAPDH, using the dinoflagellate Prorocentrum minimum as a control alga.

2.4 Discussion

Previous studies have shown that when *C. helgolandicus* is fed on *S. marinoi*, egg hatching success is impaired after 3 days of maternal feeding, with the production of apoptotic teratogenic nauplii that do not develop to adulthood (Buttino, *et al.*, 2004). Our results indicate that a diet of *S. marinoi* affects α- and β- tubulin gene expression levels in adult females after only two days of feeding by possibly reducing MT subunits and microtubule filament formation, with the cascading effect of altering pronuclear migration, DNA replication and mitotic events. Alternatively, PUAs and other oxylipins may affect protein and organelle transport along microtubules, impairing cellular

homeostasis. Ingestion of *S. marinoi* induced a pronounced down regulation of tubulin genes, with about a 3-fold down-regulation of β-tubulin and 2-fold down-regulation of α-tubulin. By contrast, diets of the dinoflagellate *P. minimum* and the flagellate *R. baltica* induced no significant up- or down-regulation of these genes.

The best RGs for *C. helgolandicus* fed on the diatom *S. marinoi*, the dinoflagellate *P. minimum* and the flagellate *R. baltica* were S20, S7 and GAPDH. These results confirm the stability of the ribosomal protein S20 as reported for the salmon louse *L. salmonis* (Frost, Nilsen, 2003) even if this species belongs to a different copepod order. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) which is widely used in gene expression studies (Hansen, *et al.*, 2008a; Spinsanti, *et al.*, 2006; Toegel, *et al.*, 2007) has been shown to be up- or down-regulated in various conditions (Bustin, 2000). In our analysis GAPDH scored third. Gene expression profiles of Elongation factor (EFA), selected as RG in *C. finmarchicus* (Hansen, *et al.*, 2008a; Hansen, *et al.*, 2008b; Hansen, *et al.*, 2009) and *L. salmonis* (Kvamme, *et al.*, 2004; Skern-Mauritzen, *et al.*, 2009), and of β-actin (ACT), used as RG in *Tigriopus japonicus* (Lee, *et al.*, 2008; Lee, *et al.*, 2007; Seo, *et al.*, 2006a), were less stable in our test conditions and were therefore considered invalid for this analysis. Histone 3 (IST), Ubiquitin (UBI), ATP synthase (ATPs) and ribosomal protein 18S gene expression levels were less stable as well. In general, our results confirm that reference genes are not universal for a given species but may change depending on experimental conditions.

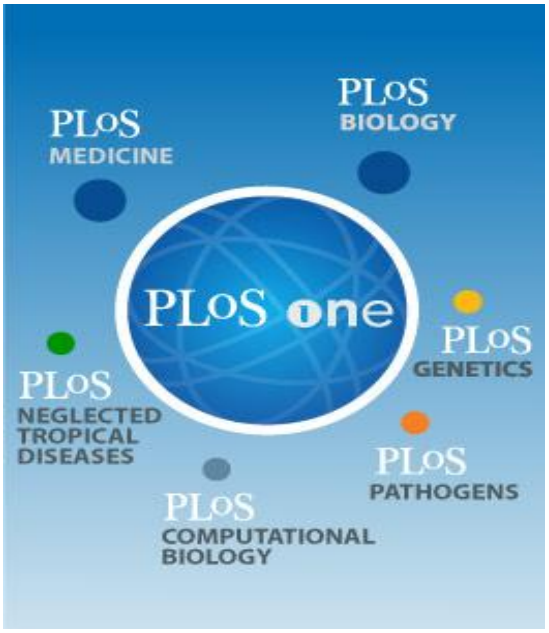
Interestingly, α- and β- tubulin subunits have also often been selected as putative RGs to normalize qPCR data (Andersen, *et al.*, 2004; Carvalho, *et al.*; Heckmann, *et al.*, 2006; Ransbotyn, Reusch, 2006; Sirakov, *et al.*, 2009), but in our experiments their expression levels were evidently affected by the oxylipin-producing diatom SKE.

Several studies have shown that the functioning of tubulin is affected by exposure to toxic compounds (Chavez, *et al.*, 2010; Lee, *et al.*, 2009), but these results have usually been obtained in humans. For example, exogenous exposure to another aldehyde, 4-hydroxy-2(E)-nonenal (HNE), in human monocytic THP-1 cells induced HNE-tubulin alpha-1B chain adducts leading to inhibition of tubulin polymerization (Chavez, *et al.*, 2010). Prenatal exposure to cocaine causes cyto-architectural alterations in the developing neocortex inducing impairments in fetal brain development and RT-qPCR analyses (Lee, *et al.*, 2009) and the down-regulation of cytoskeleton-related genes such as alpha 3d tubulin and alpha 8 tubulin in neural and/or A2B5+ progenitor cells.

Our results support, for the first time at the molecular level, previous findings by Buttino *et al*. (Buttino, *et al.*, 1999) and Hansen *et al*. (Hansen, *et al.*, 2004), that PUAs inhibit tubulin-polymerization affecting proper spindle formation and cell division. Future studies on the biological effects of these molecules should address whether the functioning of other important proteins are altered as well. Our results may help explain why recruitment processes in copepods during diatom blooms may be compromised with possible effects on cohort size of the next generation.

Chapter 3:

Molecular Evidence of the Toxic Effects of Diatom Diets on Gene Expression Patterns in Copepods



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Molecular Evidence of the Toxic Effects of Diatom Diets on Gene Expression Patterns in Copepods. PLoS One, 6; 10

3.1 Brief Introduction

The aim of the present chapter was to further explore the toxic effects of diatoms on copepod females at the gene level under two different experimental conditions: when females were fed for 2 days (2d) on *S. marinoi* compared to when they received a diet of another diatom *Chaetoceros socialis* (*C. socialis*) that does not produce PUAs and synthesizes only low levels of other oxylipins (Fontana, *et al.*, 2007b), and was thus, in theory, "less toxic" for copepods.

In addition to the previously investigated alpha and beta tubulins, here we analyzed the effects of these two diatom diets on the expression levels of genes which are known to have a primary role in generic stress responses, defense systems (e.g. aldehyde, free fatty acid and free radical detoxification) or apoptosis regulation in other organisms, from humans to marine organisms (Bouraoui, *et al.*, 2009; Einsporn, *et al.*, 2009; Hasselberg, *et al.*, 2004; Kim, *et al.*, 2008; Olsvik, *et al.*, 2009; Salazar-Medina, *et al.*, 2010; Snyder, 2000; Vasiliou, *et al.*, 2004; Wan, *et al.*, 2011) (Figure 1). We expected an activation of enzymes and proteins involved in stress responses (e.g heat shock proteins, phase I and phase II enzymes), but, in particular, we hypothesized expression level increases of enzymes that could detoxify and/or metabolize toxic diatom PUAs.

To study the generic stress response of *C. helgolandicus* to diatom toxicity, we analyzed the heat shock protein families 40 and 70 (HSP40 and HSP70, respectively). HSPs are highly conserved proteins that are activated in response to various environmental stress factors (Bierkens, 2000; Feder, Hofmann, 1999). HSP70 can be involved in the tolerance of hyperthermia, ischemia/hypoxia, resistance to hydrogen

peroxide, escape from drug-induced cell cycle arrest, tolerance to ultraviolet radiation and apoptosis (Feder, Hofmann, 1999) whereas HSP40 is often co-localized with HSP70 and plays a role in regulating the ATPase activity of HSP70 (Fan, *et al.*, 2003).

Since oxylipins induce LPO and an increase in free fatty acids (Fink, *et al.*, 2006), we also analyzed the microsomal cytochrome P450 family 4 monooxygenases (CYP4) that generally catalyze the ω-hydroxylation of fatty acids, arachidonic acid and derivatives such as leukotrines and prostanoids (Hsu, *et al.*, 2007). Cytochrome P450 (CYP) enzymes, especially members of the CYP1, CYP2, CYP3, and CYP4 families are generally involved in oxidative modification (known as Phase I reaction) of chemicals into more hydrophilic metabolites to enhance their elimination or inactivation (Goldstone, *et al.*, 2006).

Diatom oxylipins are also known to induce an increase in free radicals, such as reactive oxygen species (ROS) (Fontana, *et al.*, 2007b) which induce oxidative stress and damage to DNA, RNA, proteins, lipids and carbohydrates. We therefore analyzed enzymes involved in ROS detoxification, such as catalase (CAT) and superoxide dismutase (SOD) (Bigot, *et al.*, 2010) and antioxidant activity, such as glutathione synthase (GSH-S) and glutathione S-transferase (GST). Glutathione is an important cell scavenger molecule which facilitates dis-activation of radical compounds. GST is involved in phase II detoxification reactions, catalyzing the nucleophilic attack of glutathione on electrophilic substrates, decreasing their reactivity with cellular macromolecules, facilitating dissolution of the complex glutathione-substrate in the aqueous cellular and extracellular media and, consequently, its elimination from the body (Sheehan, *et al.*, 2001).

We also analyzed aldehyde dehydrogenases (ALDHs) which constitute a superfamily of enzymes that catalyze the oxidation of endogenous and exogenous aldehydes into their corresponding carboxylic acids. These enzymes are generally involved in many processes including amino acid catabolism, neurotransmitter metabolism, xenobiotic and drug biotransformation, protection from osmotic stress and detoxification reactions (Vasiliou, *et al.*, 2004; Yoshida, *et al.*, 1998). We selected several aldehyde dehydrogenase isoforms (ALDH2, ALDH3, ALDH6, ALDH7, ALDH8 and ALDH9) that are mainly involved in aldehyde detoxification due to lipid peroxidation (LPO) (Brocker, *et al.*, 2010; Marchitti, *et al.*, 2008; Vasiliou, *et al.*, 2004).

Previous studies have shown that PUAs and oxylipins also induce apoptosis and teratogenesis in the offspring of female copepods that have fed on diatoms for ≥ 5d (Ianora, *et al.*, 2004). We therefore determined the transcription level of a protein belonging to the Inhibitor of apoptosis family (IAP), the cell cycle and apoptosis regulatory 1 protein (CARP) and the cellular apoptosis susceptibility protein (CAS). IAP levels increase in certain tumors probably contributing to resistance to apoptosis (Lederman, *et al.*, 2008), CAS and CARP are also both involved in apoptosis (Brinkmann, 1998; Kim, *et al.*, 2008; Tai, *et al.*, 2010): CARP is a novel cell growth regulator (Rishi, *et al.*, 2006) and CAS is necessary in the mitotic spindle checkpoint that ensures genomic stability during cell division (Tai, *et al.*, 2010). In addition, we also analyzed microtubule subunits (alpha and beta tubulins), necessary for mitotic spindle formation. Microtubules (MTs) have many other cellular functions including development and maintenance of cell shape, growth, signaling, protein movement, intracellular vesicle transport and organization and positioning of membranous organelles (Calligaris, *et al.*, 2010; Harrison, *et al.*, 2009; Jordan Mary Ann, 2004; Nogales, *et al.*, 1998).

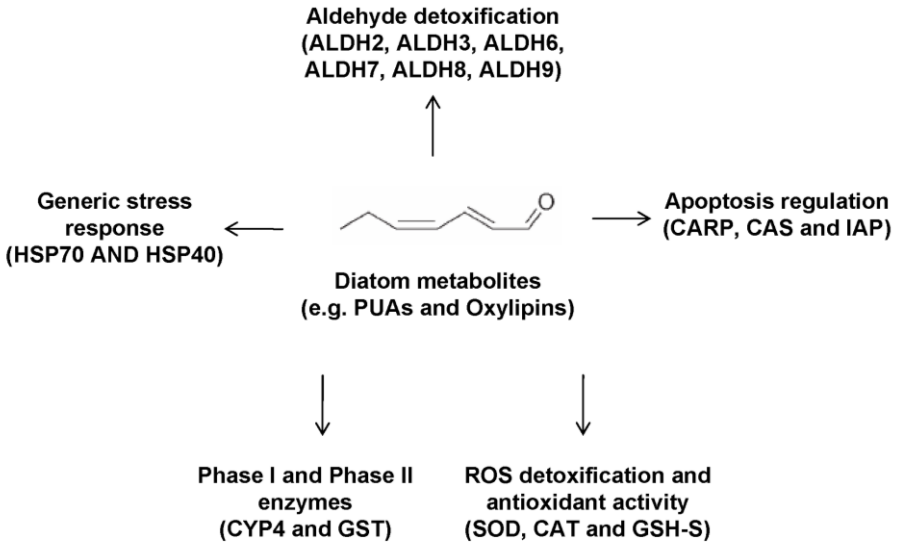

Figure 3.1. Putative systems affected by diatom metabolites in the copepod *Calanus helgolandicus.* A synopsis of the defense and detoxification systems and generic stress response (with selected genes in parenthesis) studied in *C. helgolandicus* females exposed to different diatom diets (*Skeletonema marinoi* and *Chaetoceros socialis*). Selected genes were two HSPs (HSP70 and HSP40), six Aldehyde dehydrogenases (ALDH2, ALDH3, ALDH6, ALDH7, ALDH8, ALDH9), Cytochrome P450-4 (CYP4), Catalase (CAT), Superoxide Dismutase (SOD), Glutathione S-Transferase (GST), Glutathione Synthase (GSH-S), 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).

3.2 Methods

3.2.1 Microalgae culture

The planktonic diatoms *Skeletonema marinoi* (SMFE6; Adriatic Sea isolate FE6) and *Chaetoceros socialis* (CSFE17) were cultured as described in (Gerecht, *et al.*, 2011) and harvested during the stationary growth phase. Both species are part of the culture collection at the SZN. SMFE6 produces the PUAs 2-trans-4-cis-hepta-2,4-dienal as the

dominant compound with smaller quantities of 2-trans-4-cis-octa-2,4-dienal and 2-trans-4-cis-octa- 2,4,7-trienal as well as a number of other products deriving from the oxidation of fatty acids including 9S-hydroxy-hexadecatrienoic acid, 11,9-hydroxy-epoxy-hexadienoic acid, 9S-hydroxy-hexatetraenoic acid, 5R- and 15S-hydroxy-eicosapentaenoic acids and 13,14S-hydroxy-epoxy-eicosatetraenoic acid, as described in (Fontana, *et al.*, 2007b; Gerecht, *et al.*, 2011). CSFE17 produces 9S-hydroxy-eicosapentaenoic acid, 9S-hydroperoxy-eicosapentaenoic acid and 7,8-hydroxy-epoxy-eicosatetraenoic acid but not PUAs (Fontana, *et al.*, 2007b).

3.2.2 Copepod Feeding Experiments

Calanus helgolandicus specimens were collected in the North Adriatic Sea and transported to Naples where they were placed in a 500 L re-circulating copepod breeding system (Buttino, *et al.*, 2011). *C. helgolandicus* adult females were isolated under a Leica stereomicroscope, transferred to 1000 ml bottles (about 15-20 copepods/bottle) filled with 0.22 µm filtered sea water (FSW) at 20°C and fed either unialgal diets of the control flagellate *Rhodomonas baltica* (7500-8000 cells/ml) (which does not produce any oxylipins) or the test diatoms *S. marinoi* (45.000-60.000 cells/ml) and *C. socialis* (48.000-55.000 cells/ml) for two days (2d). After 2d, copepods were transferred to clean bottles with FSW for 24h to eliminate any algal residues in the gut. For each diet, triplicate samples of 5 animals each were carefully transferred to 500 µl Trizol Reagent (Invitrogen), frozen directly in liquid nitrogen and stored at -80°C until RNA extraction.

3.2.3 RNA extraction and cDNA synthesis

Total RNA was extracted using a modified Trizol manufacturer's protocol (Invitrogen) (For more details see Appendix 1). Each sample was treated with DNaseI (Invitrogen) according to the instruction manual to remove hypothetically contaminating DNA. RNA quantity and purity was assured by Nano-Drop (ND-1000 UV-Vis spectrophotometer; NanoDrop Technologies), RNA quality by gel electrophoresis. 1 µg of each RNA sample was retro-transcribed in complementary DNA (cDNA) (doublestrand DNA version of an mRNA molecule) 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, 1µl 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 at 85°C for 5 min.

3.2.4 Primer design

Primers were designed considering the alignment of conserved domains in other species such as *Calanus finmarchicus, Calanus californicus, Tigriopus japonicus, Homarus americanus, Drosophila melanogaster* and *Anopheles gambiae*. Alignments were performed with Clustal W (Clustal) and BioEdit (BioEdit). Gene Runner, V3.05 (Hasting Software) was used to predict primer melting temperature (T_m) and check if primers formed dimers, hairpin, bulge and internal loops. Table 3.1 lists primers' sequences, amplicon size, correlation coefficient (R^2) and efficiency (E). PCR conditions were optimized on a GeneAmp PCR System 9700 (Perkin Elmer) (for more

details see chapter 1, paragraph 2.2.4). The amplicons that had one-single band on the agarose gel were considered valid to proceed with the Reverse Transcription-Quantitative Real Time Polymerase Chain Reaction (RT-qPCR). Amplified PCR product sequences are deposited in GenBank under the Accession Numbers shown in Table 3.1.

3.2.5 Reverse Transcription-Quantitative Real Time Polymerase Chain Reaction (RT-qPCR)

The fluorescent dye SYBR GREEN was used to evaluate expression levels of the selected genes by RT-qPCR. Fluorescence was monitored once per cycle after product extension and increased above background fluorescence at a cycle number that depended on the initial template concentration (For protocol details see APPENDIX 2).

Expression levels of each target gene in the tested experimental conditions (animals fed on *S. marinoi* and *C. socialis*) were compared to the control condition (animals fed on *R. baltica*) using the REST tool (Relative expression software tool) (Pfaffl, *et al.*, 2002). Data were normalized using the ribosomal protein S20, which had previously been identified as the best reference gene under different experimental conditions (Lauritano, *et al.*, 2011a). In the present analysis, the ribosomal protein S20 was confirmed to be stable, showing a variability always lower than ± 1 cycle. The 1 x-fold expression level was therefore chosen as the threshold for significance of target genes. However, to validate our results, a statistical analysis was also performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA).

	Gene name	Acc. no.	Primer sequence 5'-3' (forward and reverse)	Amplicon size	E	R^2
			Reference gene			
S20	Ribosomal protein S20	HQ270531	CGTAAGACTCCTTGTGGTGAGG GAAGTGATCTGCTTCACGATCTC	113	89%	0.9915
			Genes of interest			
ATUB	Alpha tubulin	HQ270529	ACAGCTTCTCCACCTTCTTCTC GTTGTTGGCGGCATCCTC	167	94%	0.9997
BTUB	Beta tubulin	HQ270528	GGATTTCAGCTGACCCACTC GTCTCATCAGTATTTTCCACCAG	205	97%	0.9862
CYP4	Cytochrome P450-4	JF825512	CTGATCACTCCAACTTTTCACTTC CCATTGCAGTCTCACAGATTATG	169	100%	0.959
ALDH2	Aldehyde Dehydrogenase 2	JF825506	GGACAAGGCAGATGTCAACAA ATAGGGTTTGCCATTGTCAAG	181	100%	0.998
ALDH3	Aldehyde Dehydrogenase 3	JF825507	CCTCTTGGTGTTGTCCTGATC CCAACTCTGATGGCTTGATG	117	95%	0.997
ALDH6	Aldehyde Dehydrogenase 6	JF825508	GAGCAGTGCTGCAGCAACAC GGAACATCCAGAGGGGGATC	164	100%	0.989
ALDH7	Aldehyde Dehydrogenase 7	JF825509	CAGGAGTATGTTGACATCTGTGAC GAAGTTGAAGGCGGTGATG	154	100%	0.988
ALDH8	Aldehyde Dehydrogenase 8	JF825510	CTGGAGGAGTTTGCAGTGG GCCAGCCACACCAATAGG	198	100%	0.997
ALDH9	Aldehyde Dehydrogenase 9	JF825511	GGAAAACCAATCTGGGAAGC CAAAGGGTAGTTCCAGGCTC	183	100%	0.988
GST	Glutathione S-Transferase	JF825513	CAACCCCCAGCACACTGTG GGATAGACACAATCACCCATCC	210	83%	0.992
GSH-S	Glutathione Synthase	JF825516	GAGAAGGCAAAGGACTATGCTC GGCAACCTTGTGCATCAAC	180	97%	0.998
CAT	Catalase	JF825517	TGTACATGCAAAGGGAGCTG GGTGTCTGTTTGCCCACTTT	104	100%	0.998
SOD	Superoxide Dismutase	JF825518	GGAGATCTTGGCAATGTTCAG CAGTAGCCTTGCTCAGTTCATG	166	97%	0.991
CAS	Cellular Apoptosis Susceptibility Protein	JF825520	CTACAACCACTACCTGTTCGAGT CAGGGACATGATCTGGAACAC	169	100%	0.995
CARP	Cell Cycle and Apoptosis Regulatory 1 Protein	JF825519	GCCAAGAGTGGGAAGTTTGAC GAACATTTCATTGAACAATTCTGC	126	98%	0.997
IAP	Inibitor of Apoptosis Protein	JF825521	CAGGATTCTTCTACACAGGCAG CCATTTCTTGTGTTCTCCCC	108	100%	0.988
HSP70	Heat Shock Protein 70	JF825515	CTTCGTTTGGTATCCATGTTGGTA CTCTGTGTCCTGGTAGGCGAC	130	100%	0.997
HSP40	Heat Shock Protein 40	JF825514	GGATTATTATAAAGTGCTGGGG GTCACTAAGTACATCATAGGCCTC	163	100%	0.996

Table 3.1. Reference Gene and Genes of Interest in the copepod *Calanus helgolandicus* RT-qPCR assays. Table 3.1 shows Pubmed accession numbers, primer sequences, amplicon sizes (base pair), oligo efficiencies (E) and correlation factors (R^2) of the reference gene and genes of interest.

3.3 Results

The effects of diatom diets on *C. helgolandicus* females were evaluated by analyzing expression levels of genes involved in generic stress responses, defense systems, aldehyde detoxification or apoptosis regulation in other organisms (Figure 3.1). *C. helgolandicus* females fed the strong oxylipin and PUAs-producing diatom *S. marinoi* showed a general pattern of reduction in the expression levels of almost all the selected genes compared to females fed the control flagellate *R. baltica* (Figure 3.2, 3.3, 3.4). Both HSP40 and HSP70 transcript levels were reduced even if the change was only significant for HSP40 (p value<0.01, students't-test, GraphPad Software) (Figure 3.2). Expression levels of these genes in animals fed *C. socialis* did not change significantly. Enzymes involved in phase I and phase II reactions and anti-oxidant activity (CYP4, GST, GSH-S, CAT and SOD) did not show any significant changes in their expression levels in animals fed both diatom diets.

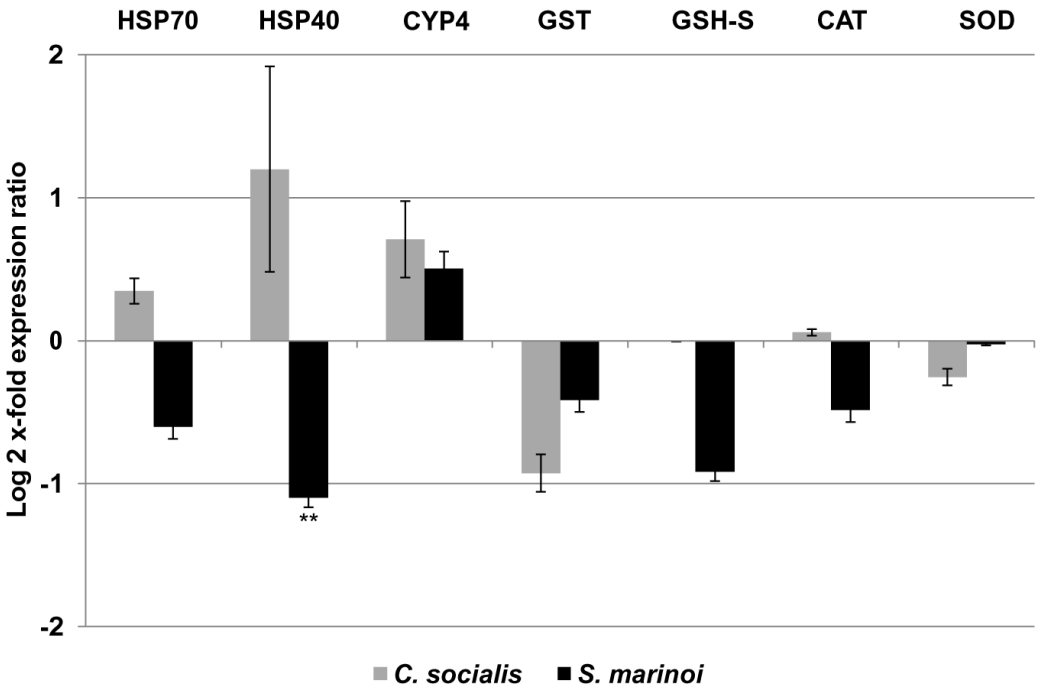

Figure 3.2. Expression levels of genes involved in stress and defense systems in the copepod *Calanus helgolandicus*. Changes in expression levels of Heat shock protein 70 (HSP70) and 40 (HSP40), Cytochrome P450-4 (CYP4), Glutathione S-Transferase (GST), Glutathione Synthase (GSH-S), Catalase (CAT) and Superoxide Dismutase (SOD) genes in *C. helgolandicus* fed either unialgal diets of *Skeletonema marinoi* (*S. marinoi*) or *Chaetoceros socialis* (*C. socialis*) compared to expression levels in females fed on the control *Rhodomonas baltica* (represented in the figure by x-axis) (** with p value<0.01, students't-test, GraphPad Software). The ribosomal protein S20 was used as reference gene to normalize the data.

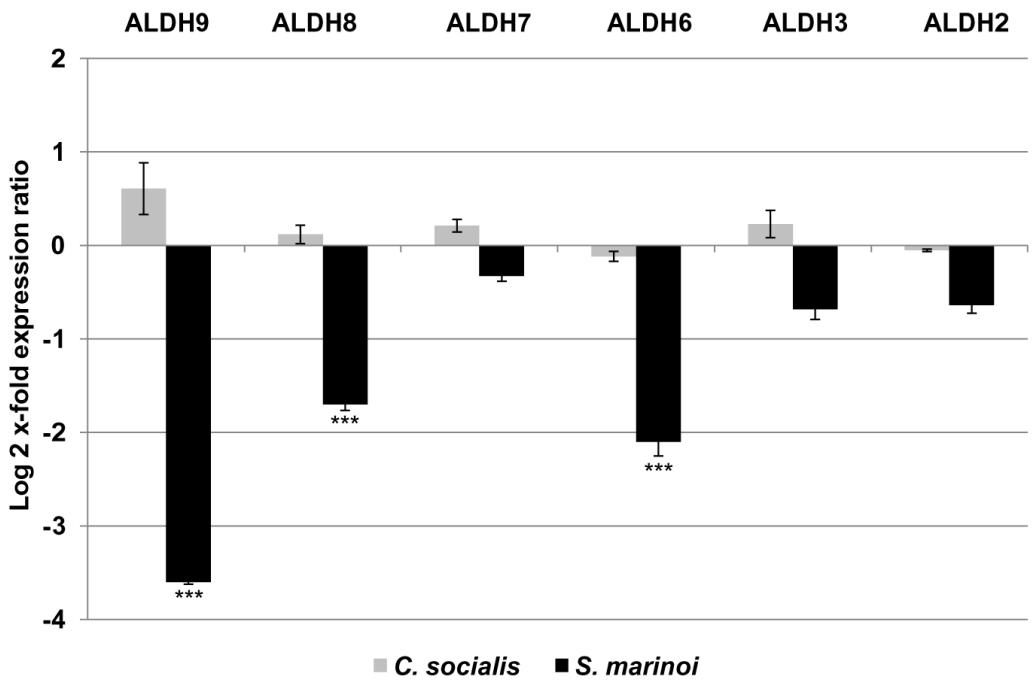

Figure 3.3. Relative gene expression levels of aldehyde dehydrogenases (ALDH) in the copepod *Calanus helgolandicu*s. Changes in ALDH2, ALDH3, ALDH6, ALDH7, ALDH8 and ALDH9 gene expression levels in *C. helgolandicus* females fed either unialgal diets of *Skeletonema marinoi* (*S. marinoi*) or *Chaetoceros socialis* (*C. socialis*) compared to expression levels in females fed on the control *Rhodomonas baltica* (represented in the figure by x-axis) (*** with p value<0.001, students't-test, GraphPad Software). The ribosomal protein S20 was used as reference gene to normalize the data.

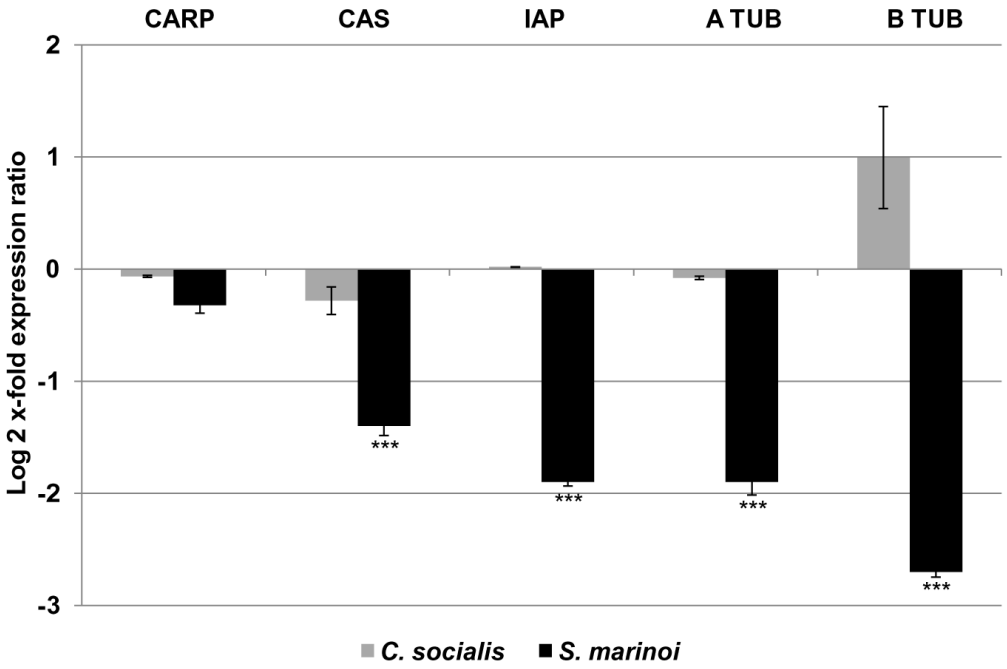

Figure 3.4. Expression analysis of genes involved in apoptosis and mitotic spindle formation in *C. helgolandicus*. Changes in expression levels of Cell Cycle and Apoptosis Regulatory 1 Protein (CARP), Cellular Apoptosis Susceptibility Protein (CAS), Inhibitor of Apoptosis Protein (IAP), and Alpha and Beta tubulins (ATUB and BTUB) genes in *C. helgolandicus* fed either unialgal diets of *Skeletonema marinoi* (*S. marinoi*) or *Chaetoceros socialis* (*C. socialis*) compared to expression levels in females fed on the control *Rhodomonas baltica* (represented in the figure by x-axis) (*** with p value<0.001, students't-test, GraphPad Software). The ribosomal protein S20 was used as reference gene to normalize the data.

On the contrary, all six ALDH isoforms (ALDH2, ALDH3B1, ALDH6, ALDH7, ALDH8, and ALDH9) had lower expression levels in *C. helgolandicus* fed *S. marinoi* than those fed the control diet (Figure 3.3). However, only ALDH6, ALDH8 and ALDH9 were significantly affected, showing a 2-3 fold reduction in their expression levels (p value<0.001, students't-test, GraphPad Software). On the contrary, the expression levels for the same genes in copepods fed *C. socialis* did not show significant changes, indicating that these genes were not affected by this diatom diet.

Of the three proteins involved in apoptosis regulation (CARP, CAS and IAP) CAS and IAP expression levels were strongly reduced by *S. marinoi* compared to the control *R. baltica* and to *C. socialis*. In particular, CAS and IAP showed a significant 2-fold reduction (p value<0.001, students't-test, GraphPad Software) (Figure 3.4), while changes in CARP expression levels were close to zero. Gene expression profiles of alpha and beta tubulins, essential proteins for mitotic spindle formation, did not vary significantly in copepods fed on *C. socialis* compared to the control *R. baltica*. In contrast, *C. helgolandicus* fed *S. marinoi* showed a significant reduction of about 2-fold for alpha tubulin and 3-fold for beta tubulin (p value<0.001, students't-test, GraphPad Software) (Figure 3.4).

3.4 Discussion

Our results show that two days (2d) of feeding of *C. helgolandicus* on *S. marinoi* is sufficient to inhibit a series of genes involved in generic stress response, aldehyde detoxification and apoptosis regulation. Of the analyzed transcripts at least 50% were strongly reduced (ALDH9, ALDH8 and ALDH6, CAS, IAP, HSP40, alpha- and beta-tubulin) with a *S. marinoi* diet, while no significant gene expression changes were observed in animals fed on the other diatom *C. socialis*. Previous studies have shown that after 2d of feeding on *S. marinoi* egg viability in *C. helgolandicus* is still high (>90%) and decreases to about 50% after 3d (Fontana, *et al.*, 2007b). On the contrary, with a *C. socialis* diet, egg viability is high (90%) even after 3d (Fontana, *et al.*, 2007b) indicating that this diatom is less toxic for copepod reproduction. Fontana and co-workers (Fontana, *et al.*, 2007b) concluded that the lower toxicity of *C. socialis* was due to the fact that this diatom does not produce PUAs but only low quantities of hydroxyl-acids and epoxy-alcohols compared to *S. marinoi*. Our results indicate that there is a significantly different response in gene expression patterns in *C. helgolandicus* fed on these two diets thereby offering a possible explanation as to why in nature certain diatom blooms may be more toxic for copepods (Ianora, *et al.*, 2004; Koski, 2007; Miralto, *et al.*, 1999) compared to others (Irigoien, *et al.*, 2002; Koski, 2007).

Until now, gene expression studies in copepods have been performed after exposure to various toxicants such as naphtalene (Hansen, *et al.*, 2008b), diethanolamine (Hansen, *et al.*, 2010) and mono ethanol amine (MEA), water-soluble fractions of oil (WSFs), trace metals (Hansen, *et al.*, 2007) and endocrine-disrupting

chemicals (Lee, *et al.*, 2006) (as reviewed by (Lauritano, *et al.*, 2011c). In most of these studies (Hansen, *et al.*, 2008a; Hansen, *et al.*, 2009; Hansen, *et al.*, 2011; Lee, *et al.*, 2008), detoxification gene expression levels increased when copepods were challenged with toxicants, but in our case there was a general pattern of decrease and both general stress systems and specific responses seemed to be inhibited.

Both HSP70 and HSP40 expression levels decreased in females fed on *S. marinoi* suggesting a reduction in chaperone activity in the folding of new proteins, repairing of unfolded and damaged proteins, and inhibition of protein aggregations, thereby leading to an increase in cellular damage. Romano et al. (Romano, *et al.*, 2011) have recently shown that sea urchins activate HSP70 when challenged with low concentrations (0.25 µg/ml) of the PUA decadienal thereby protecting embryos against the toxic effects of this aldehyde. This up-regulation was only found at 9 h post fertilization (hpf), while at 5, 24 and 48 hpf, expression levels were comparable to the control. Small changes in HSP70 mRNA levels were found in *C. finmarchicus* after naphtalene exposure (Hansen, *et al.*, 2008b), while Rhee and co-workers (Rhee, *et al.*, 2009) showed a concentration-dependent increase in the expression of HSP70 transcripts after exposure to trace metals (i.e. copper, silver, and zinc), with an increase caused by bisphenol A (BPA) and a decrease by 4-nonylphenol (NP) and 4-t-octylpheno (OP).

Enzymes involved in antioxidant cell activity (GST and GSH-S) and in free radical detoxification (CAT and SOD) did not show significant expression level changes in *C. helgolandicus* fed either of the two diatom diets indicating that they were not involved in the defense response of this copepod species, at least after two days of exposure. Kozlowsky-Suzuki and co-workers (2008) also suggested that GST

enzymatic activity did not seem to play a role in detoxification of copepods exposed to toxic dinoflagellate algae: *Alexandrium minutum* and *Alexandrium tamarense*, which contained Paralytic Shellfish Poisoning (PSP) toxins, and the dinoflagellate *Prorocentrum lima* with Diarrhetic Shellfish Poisoning (DSP) toxins. On the contrary, GST expression levels were affected in the copepod *Calanus finmarchicus* after exposure to naphthalene (Hansen, *et al.*, 2008b) and diethanolamine (DEA) (Hansen, *et al.*, 2010) and in the copepod *Tigriopus japonicus* exposed to trace metals and hydrogen peroxide (H_2O_2) (Lee, *et al.*, 2008). The responses were mainly concentration- and time-dependent and varied with the tested stressors. Hansen and co-workers (Hansen, *et al.*, 2008b) showed that only the lowest naphthalene concentration in *C. finmarchicus* led to increased mRNA levels of the ROS detoxification enzymes SOD and CAT, but no effects were found at medium and high concentrations, indicating no clear evidence for general cellular oxidative stress following naphthalene exposure. On the other hand, the transcription levels of the antioxidant glutathione synthase (GSH-S) and Cu/Zn-superoxide dismutase (SOD) changed with a concentration-dependent pattern following exposure to DEA in the same copepod species (Hansen, *et al.*, 2010). SODs expression levels in the harpacticoid copepod *Tigriopus japonicus* increased only at the highest heavy metal concentrations tested and showed different responses to endocrine disruptor chemicals (EDCs) depending on the specific stressor and its concentration (Kim, *et al.*, 2011).

The aldehyde dehydrogenase family, which should detoxify and inactivate aldehydes, was almost switched off in animals fed on the *S. marinoi* diet. Gene expression levels were significant reduced by about 4-fold for ALDH9 and 2-fold for ALDH8 and ALDH6. Until now, ALDH gene expression levels have been mostly

analyzed in humans and this is the first time that they have been analyzed in a copepod species. It is widely known that ALDH are involved in protecting cells from the deleterious effects of xenobiotics and endogenous aldehydes such as those derived from lipid peroxidation (Canuto, *et al.*, 1994). Our results suggest that this enzyme family in copepods fed on the diatom *S. marinoi* is probably not able to detoxify high levels of toxic diatom aldehydes, and that therefore there is accumulation of these compounds in body tissues or formation of adducts.

Selected apoptosis regulatory proteins were also affected by the *S. marinoi* diet. The two proteins whose function in humans is to inhibit apoptosis, CAS and IAP, were significantly down-regulated by about 2-fold, yet apoptotic processes were not inhibited, at least after 2d of feeding. The fact that CARP, a protein generally associated with an increase in apoptosis (Majumdar, *et al.*, 2007), did not respond to the diet suggests that there was no clear apoptosis induction in adult females in our experimental conditions. Buttino *et al*. (Buttino, *et al.*, 2008) using aldehyde-encapsulating liposomes observed apoptotic regions in copepod female gonads only after 9d of feeding. We therefore assume that induction of pro-apoptotic proteins may only occur after longer exposure to the toxic diet.

Alpha and beta tubulins, structural subunits of MTs and the targets of many natural toxins, were previously reported to be 2-fold and 3-fold down-regulated, respectively, with a *S. marinoi* diet (Lauritano, *et al.*, 2011a). Here we confirm our previous findings and also show that *C. socialis* does not induce the same pronounced reduction in the expression levels of these two genes. Future studies on PUAs-tubulin interactions may clarify if alpha and beta tubulins are the targets of toxic *S. marinoi* metabolites or if their gene expression reduction is a secondary effect of PUAs toxicity.

Our results provide molecular evidence for the toxic effects of certain diatom diets on grazers, showing that primary defense systems that should be activated to protect copepods against dangerous algae are inhibited. This exploratory study is currently being extended with the creation of a suppression subtractive hybridization library for *Calanus helgolandicus* which may further help to clarify which genes are differentially expressed in response to the ingestion of some diatom species. Given the importance of diatom blooms in nutrient-rich aquatic environments these preliminary results offer a plausible explanation for the inefficient use of a potentially valuable food resource, the spring diatom bloom, by some zooplankton (Hansen, *et al.*, 2008b; Ianora, *et al.*, 2004; Miralto, *et al.*, 1999). Also terrestrial plants produce toxins which cause abortions, reproductive dysfunction and occasional birth defects when ingested by certain grazers (Green, *et al.*, 2010) suggesting that interactions among organisms are regulated by similar mechanisms in terrestrial and marine ecosystems.

Chapter 4:

Gene expression patterns in Atlantic and Mediterranean *Calanus helgolandicus* populations feeding on the same toxic diatom diet.

In preparation as:

Lauritano Chiara, Carotenuto Ylenia, Procaccini Gabriele, Miralto Antonio and Ianora Adrianna.
Gene expression patterns in Atlantic and Mediterranean *Calanus helgolandicus* populations feeding on the same diatom diet. *In preparation*.

4.1 Brief introduction

In recent years, many studies have focused on the effects of toxicants on aquatic ecosystems and it is now generally accepted that responses to toxicants tend to be species-specific (Paoletti, 1999). It has been suggested that species which tolerate a high degree of natural abiotic stress may be pre-adapted to tolerate pollution stress as well. The copepod *Tigriopus japonicus* species, for example, inhabits rock pools of the intertidal zone of seashores in temperate and subtropical regions such as Korea, Japan and China. As these regions experience extreme changes, especially in terms of water temperature (0-40°C) and salinity (0-70 ppt), *T. japonicus* may possibly have evolved a singular cellular defense system to adapt to these environmental changes (Seo, *et al.*, 2006b). However, populations of the same species may differ in their response to toxic molecules depending on their history of exposure to these compounds. Pre-exposure of the aquatic oligochaete *Sparganophilus pearsei* to mercury in their native sediments influenced the resistance levels recorded during laboratory mercury exposures (Vidal, Horne, 2003). The effects of the dinoflagellate *Alexandrium fundyense (A. fundyense)* on egg production of two geographically separated populations (Maine and Connecticut) of the copepod *Acartia hudsonica* were examined in common environment experiments. The location in Maine regularly experiences toxic blooms of *Alexandrium* sp. whereas the location in Connecticut does not. During a 6 day period, *A. fundyense* reduced the egg production rates of the Connecticut copepod population, but not of the Maine population suggesting a local adaptation to toxic food (Zheng, *et al.*, 2011).

It is not clear if differences in the response mechanisms to stressors can also be detected among distinct populations of the same species, living in areas with a different exposure to environmental stress.

We therefore decided to analyze the effects of ingestion of the oxylipin-producing diatom *S. marinoi* on gene expression levels in two different *Calanus helgolandicus* populations: the English Channel (NE Atlantic Ocean) and Adriatic Sea (Mediterranean) populations. The two populations live in different environments, are subjected to different temperature and salinity conditions, and exposed to different diatom blooms in terms of species composition (Bonnet, *et al.*, 2005; Miralto, *et al.*, 1999; Poulet, *et al.*, 2006). Both *Calanus* populations are exposed in nature to the presence of the diatom *S. marinoi*; in the Adriatic Sea it is known to form intense winter blooms (Miralto et al. 1999) and also commonly occurs in the NE Atlantic (Kooistra, *et al.*, 2008).

4.2 Methods

4.2.1 Copepod sampling and feeding experiments

Calanus helgolandicus specimens were collected in two different geographical locations: North Adriatic Sea and North Atlantic Ocean. *C. helgolandicus* collected in the North Adriatic Sea from March to April 2009 were transported to Naples where they were placed in a 500 L re-circulating copepod breeding system (Buttino et al., 2011). Specimens were collected in the North Atlantic Ocean in May 2010, transported to the CNRS of Roscoff (France) and transferred to 10 L tanks. In both cases, 45 adult female *C. helgolandicus* were sampled from the tanks under a Leica stereomicroscope and transferred to triplicate 1 L bottles filled with 0.22 μm filtered sea water (FSW) enriched with either unialgal diets of the control non-oxylipin producing flagellate *Rhodomonas baltica* (*R. baltica*) (7500-8000 cells/ml) or the oxylipin-producing diatom *Skeletonema marinoi* (*S. marinoi*) (45.000-60.000 cells/ml) provided ad libitum. Bottles containing copepods were maintained for 5 days in temperature controlled rooms at

20°C. To avoid settlement of diatom cells to container bottoms, bottles were gently rotated every 4h. This was not necessary with bottles containing free-swimming flagellate cells.

Both algal strains belong to the Stazione Zoologica Anton Dohrn culture collection. *R. baltica* was cultured in glass jars with 0.22 µm-filtered FSW enriched with k medium at 20^{o}C and on a 12:12 hr dark:light cycle. The diatom *S. marinoi* was cultured under the same experimental conditions but with F2 medium. Every day FSW and new food was added to each bottle at the same concentration as the day before. After 12 h, 24 h, 48 h and 120 h, triplicate sub-samples of 5 animals for each diet were collected and transferred to FSW for 24h to eliminate any algal residues in the gut. After this, each replicate was carefully transferred to 500 µl Trizol Reagent (Invitrogen), frozen directly in liquid nitrogen and stored at -80°C until DNA or RNA extraction. To study the extent to which the selected genes were differentially expressed in our experimental conditions, we extracted RNA and retro-transcribed it in cDNA (double-stranded DNA version of an mRNA molecule), which was used as a template for our molecular analyses.

4.2.2 DNA extraction and population identification

Total DNA was extracted from a pool of 5 animals for each of the two copepod populations (Atlantic Ocean and Adriatic Sea) according to Trizol manufacturer's protocol (Invitrogen) (Appendix 1). DNA quantity was assured by Nano-Drop (ND-1000 UV-Vis spectrophotometer; NanoDrop Technologies), DNA quality by gel electrophoresis. In both cases, the following primers were used to amplify a 518 bp fragment of the mitochondrial Cytochrome Oxidase subunit I region (COI): ChelgCOI-

F (5′-GGCCAAAACAGGGAGAGATA-3′) and ChelgCOI-R (5′-CGGGACTCAGTATAATTATTCGTCTA-3′) (Papadopoulos*, et al.*, 2005). 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× 2mM dNTP, 0.8 μl of 5U/μl Taq Roche, 1 μl of 20 pmol/μl for each oligo, 1,5 μl template DNA and nuclease-free water to 20 μl. The PCR program consisted of a denaturation step at 94°C for 3 min, 35 cycles at 94°C for 1 min, 50°C for 45 sec and 72°C for 1 min, 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 COI region, the resulting bands were excised from the gel and extracted according to the QIAquick Gel Extraction Kit protocol (QIAGEN) and sequence 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 analysed on the Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems). The identity of each sequence was confirmed using the bioinformatics tool BLAST (Basic local alignment search tool).

4.2.3 RNA extraction and cDNA synthesis

Total RNA was extracted using a modified Trizol manufacturer's protocol (Invitrogen) (For more details see Appendix 1). RNA quantity and purity was assured by Nano-Drop (ND-1000 UV-Vis spectrophotometer; NanoDrop Technologies), RNA quality by gel electrophoresis. 1 μg of each RNA 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, 1µl 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 at 85°C for 5 min.

4.2.4 Reverse transcription-Quantitative real time polymerase chain reaction (RT-qPCR)

The fluorescent dye SYBR GREEN was used to evaluate expression levels of the selected genes by RT-qPCR. Fluorescence was monitored once per cycle after product extension and increased above background fluorescence at a cycle number that depended on the initial template concentration (For protocol details see APPENDIX 2).

To study expression levels for each target gene relative to the most stable RG, S20 (as discussed in chapter 2, Lauritano *et al*., 2011a), we used the REST tool (Relative expression software tool) (Pfaffl, et al., 2002). Copepods fed on the dinoflagellate *Rhodomonas baltica* were used as control condition. Statistical analysis was performed using GraphPad Prim statistic software, V4.00 (GraphPad Software).

4.2.5 Egg viability

For each feeding experiment, eggs were collected after 48, 96 and 120 h for Atlantic *C. helgolandicus* females and 48, 72 and 120 h for Mediterranean *C. helgolandicus* females. Eggs were collected by filtering each bottle on a 50 µn filter. Eggs were sorted under a Leica stereomicroscope and incubated in 50 ml glass jars with

filtered sea water (FSW). After 24 h the number of hatched eggs was determined and viability calculated.

4.3 Results

4.3.1 Population identification

A 518 bp fragment of the mitochondrial Cytochrome Oxidase subunit I region (COI) was amplified for each *C. helgolandicus* population. The identity of amplified fragments was confirmed using the bioinformatics tool BLAST (Basic local alignment search tool). COI sequence in animals collected from the Adriatic Sea corresponded to the haplotype H8 published by Papadopoulos and co-workers (2005) associated with specimens living in the NE Atlantic and/or Adriatic Sea. Here the sequence was:

5′-

GGCCAAAACAGGGAGAGATAA**A**AGAAGTAAGACCGCAGTAATTAGAACAG
CCCAGGCAAAAAGAGGCATTCGATCAAG**C**AATATACCAAACACTCGAAGAT
TGCCAAGGGTTCTAATAAAATT**C**ACAGCCCCTAAAATAGATCTCACCCCAG
CTAAATGTAACGAAAAAATAGCAAAGTCGACAGAAGCTCCAGCATGGGCTA
CATTTCTGGATAGGGGGGGGTACACGGTTCACCCAGTACCTGCGCCCCTTTC
AACCAGAGATCTTGACAAAAGTATAATTAAAGCTGGCATTAAGAACCAGAA
TCTTATATTATTTATACGAGGAAATGCCATATCTGCTGCACCCAATATTAAA
GGGACCAATCAGTTTCCAAATCCTCCAATTAAAATAGGCATAACTATAAAA
AAAATTATAATAAATGCGTGTGCAGTTACTACAACGTTATATACTTGATCAT
CTCCAATTAAAGACCCAGCTTGACCTAATTCTAGACGAATAATTATACTGAG
TCCCG-3′.

COI sequence in animals collected from the Atlantic Ocean corresponded to the haplotype H1 typical of individuals of the NE Atlantic (Papadopoulos, *et al.*, 2005). COI sequence from *C. helgolandicus* collected in the Atlantic Ocean was:

5′-

GGCCAAAACAGGGAGAGATAA**G**AGAAGTAAGACCGCAGTAATTAGAACAG
CCCAGGCAAAAAGAGGCATTCGATCAAG**T**AATATACCAAACACTCGAAGAT
TGCCAAGGGTTCTAATAAAATT**T**ACAGCCCCTAAAATAGATCTCACCCCAG
CTAAATGTAACGAAAAAATAGCAAAGTCGACAGAAGCTCCAGCATGGGCTA
CATTTCTGGATAGGGGGGGGTACACGGTTCACCCAGTACCTGCGCCCCTTTC
AACCAGAGATCTTGACAAAAGTATAATTAAAGCTGGCATTAAGAACCAGAA
TCTTATATTATTTATACGAGGAAATGCCATATCTGCTGCACCCAATATTAAA
GGGACCAATCAGTTTCCAAATCCTCCAATTAAAATAGGCATAACTATAAAA
AAAATTATAATAAATGCGTGTGCAGTTACTACAACGTTATATACTTGATCAT
CTCCAATTAAAGACCCAGCTTGACCTAATTCTAGACGAATAATTATACTGAG
TCCCG-3′.

These two sequences differed only for 3 out of 518 nucleotides: in the position of nucleotides 22, 79 and 124 (in bold and underlined).

4.3.2 Expression level of genes of interest (GOI)

4.3.2.1 Adriatic *C. helgolandicus* population

Expression levels of most genes belonging to the primary defence system, or to aldehyde detoxification and apoptosis regulation did not show significant changes in *C. helgolandicus* specimens fed the oxylipin-producing diatom *S. marinoi* for 12 and 24 h except for GST, ALDH3 and HSP70 (Figure 4.1 and 4.2). After 12 h, GST was up-regulated by about 2-fold (Figure 4.1), while ALDH3 was significantly down-regulated

by almost 3-fold (p value<0.001, students't-test, GraphPad Software) (Figure 4.2). The GST increase was not statistically significant due to high replicate variability and high SD (SD=1.396). HSP70 was also significantly down-regulated after 12 h (p value<0.001, students't-test, GraphPad Software) and up-regulated after 24 h (p value<0.05, students't-test, GraphPad Software) (Figure 4.1).

After 48 h most genes were down-regulated: ALDH9, ALDH8, ALDH6, HSP40, CAS, IAP, alpha and beta tubulins (as discussed in chapter 3, Lauritano *et al*., 2011b). This down-regulation was highly significant (p value < 0.01 for HSP40 and p value < 0.001 for for ALDH9, ALDH8, ALDH6, CAS, IAP, alpha and beta tubulins, students't-test, GraphPad Software) (Figures 4.1, 4.2 and 4.3).

By contrast, expression levels of many of the analyzed genes showed a totally different pattern after 120 h of feeding on the toxic algae, compared to the control. Of the genes belonging to a more generic stress response, HSP40 and GSH-S were both up-regulated (p value<0.01 for GSH-S, students't-test, GraphPad Software), while the others did not change. ALDH9, ALDH7, ALDH3 and ALDH2 levels also increased (p value<0.05 for ALDH2, students't-test, GraphPad Software). Of these, ALDH7 was markedly up-regulated by 3-fold but with high replicate variability (SD= 2.12), while ALDH2 by 2-fold. CAS and CARP expression levels also increased, but their up-regulation was not statistically significant; IAP also did not show any significant changes.

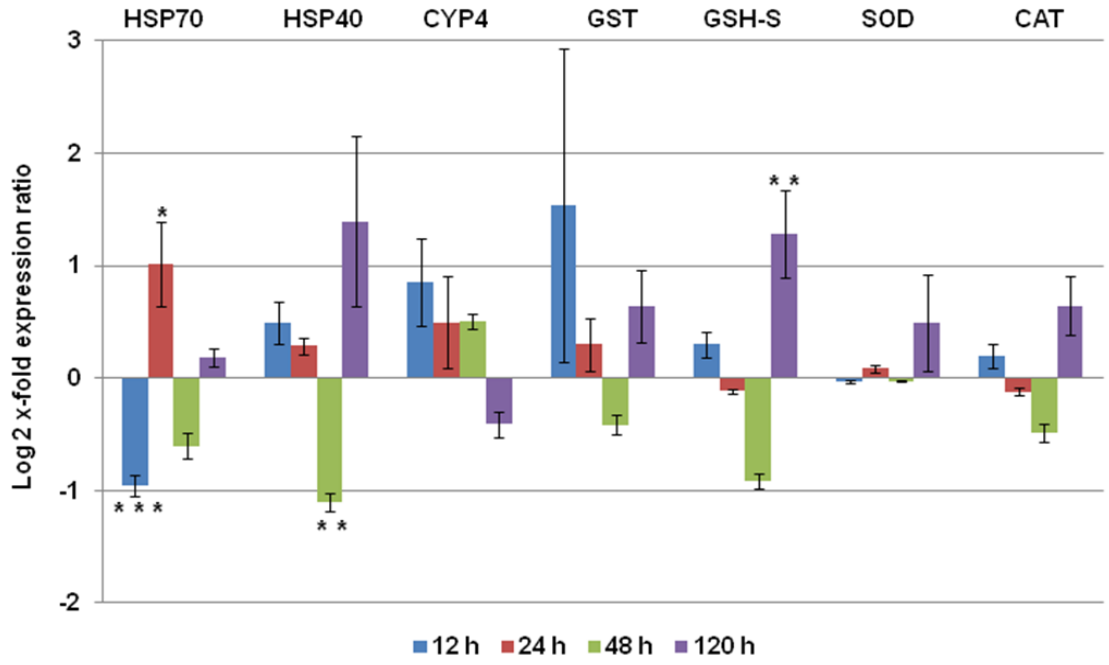

Figure 4.1. Stress response and defense system expression patterns in Adriatic *Calanus helgolandicus* females fed on the oxylipin-producing diatom *Skeletonema marinoi* (* with p value<0.05, ** with p value<0.01, *** with p value<0.001, students't-test, GraphPad Software).

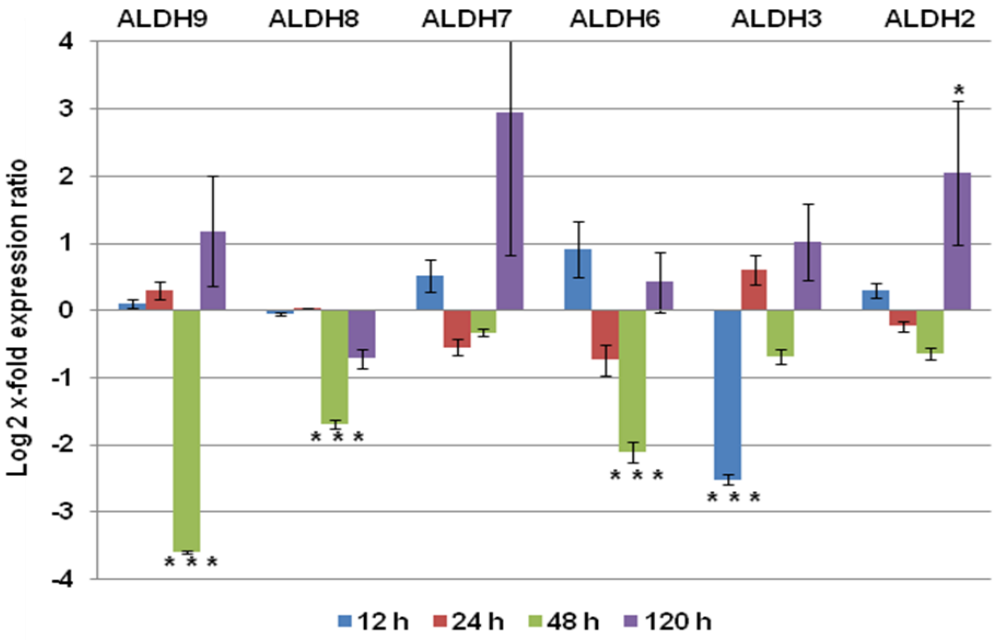

Figure 4.2. Aldehyde dehydrogenase expression levels in Adriatic *Calanus helgolandicus* females fed on the oxylipin-producing diatom *Skeletonema marinoi* (* with p value<0.05, *** with p value<0.001, students't-test, GraphPad Software).

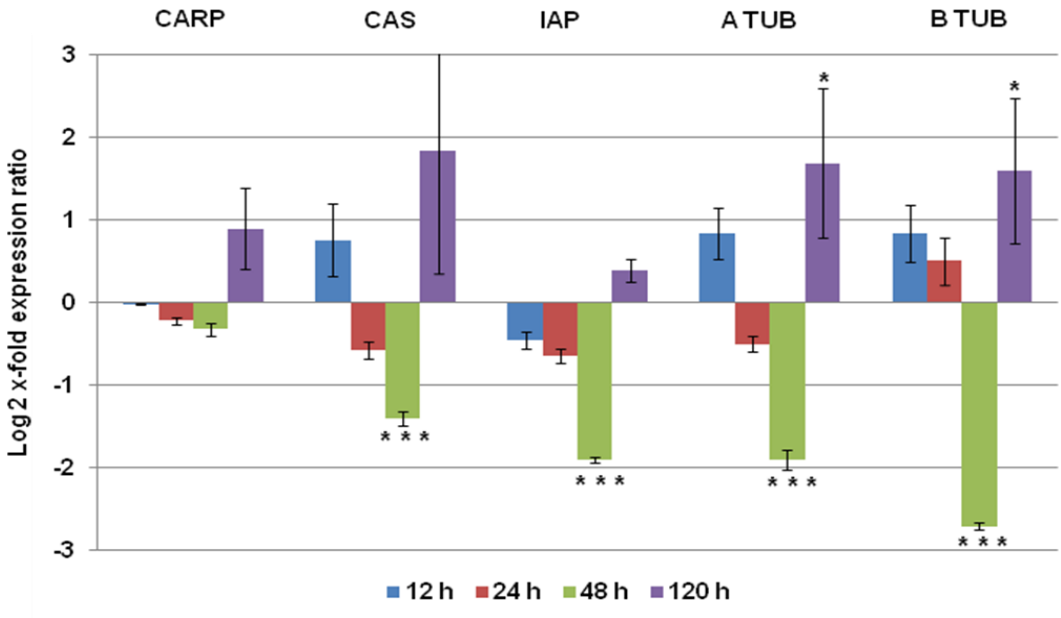

Figure 4.3. Expression levels of genes involved in apoptotis regulation and mitotic spindle formation and stability in Adriatic *Calanus helgolandicus* females fed on the oxylipin-producing diatom *Skeletonema marinoi* (* with p value<0.05, *** with p value<0.001, students't-test, GraphPad Software).

4.3.2.2 Atlantic *C. helgolandicus* population

After 12 h of feeding on *S. marinoi*, ALDH3 and HSP70 were both significantly down-regulated (p value<0.001, for both genes, students't-test, GraphPad Software), as for the Adriatic population (Figures 4.4 and 4.5). GST was significantly up-regulated by about 4-fold (p value<0.001, students't-test, GraphPad Software), as in the Adriatic population. Free radical detoxification systems were already activated after 12 h in the Atlantic compared to the Adriatic population: CAT expression levels showed a significant 2-fold increase (p value<0.001, students't-test, GraphPad Software).

After 24 h, CAT was still up-regulated (p value<0.001, students't-test, GraphPad Software) (Figure 4.4) and there was also a 2-fold increase in ALDH -6, 7 and 8, and beta tubulin (p value<0.001, for all the genes, students't-test, GraphPad Software) (Figure 5.5).

As for the Adriatic population, some genes were down-regulated after 48 h (Figures 4.4, 4.5 and 4.6) even if the down-regulation was less pronounced in the Atlantic population. HSP40, 70, CYP4 and GST were down-expressed (p value<0.001, for CYP4 and GST, students't-test, GraphPad Software) (Figure 4.4).

A completely different response was observed after 120 h. ALDH8 was significantly down-expressed by 2-fold, while GST levels significantly increased (p value<0.001, for both genes, students't-test, GraphPad Software) (Figures 4.4 and 4.5). No significant changes were observed for the other genes (Figures 4.4, 4.5 and 4.6).

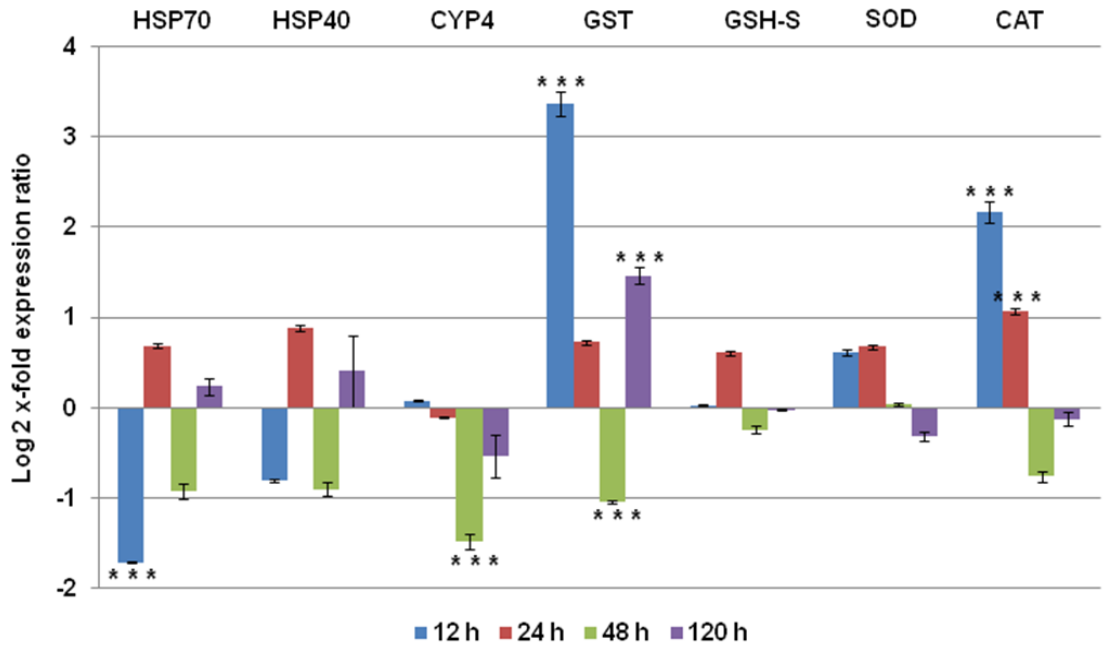

Figure 4.4. Stress response and defense system expression patterns in Atlantic *Calanus helgolandicus* females fed on the oxylipin-producing diatom *Skeletonema marinoi* (*** with p value<0.001, students't-test, GraphPad Software).

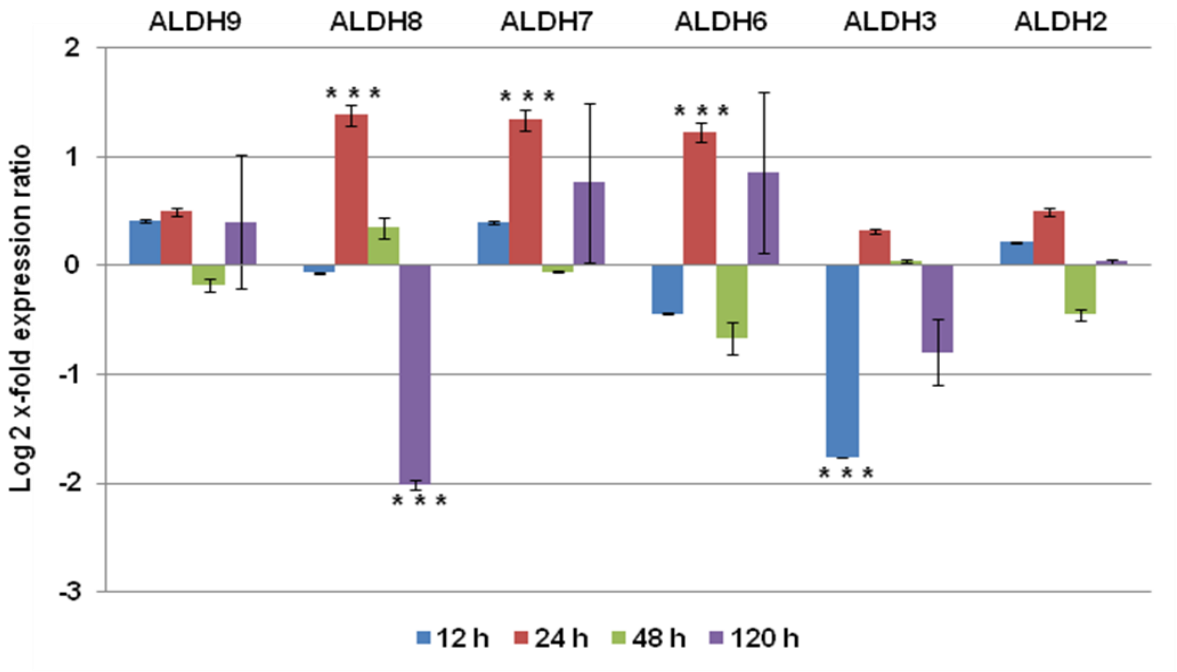

Figure 4.5. Aldehyde dehydrogenase expression levels in Atlantic *Calanus helgolandicus* females fed on the oxylipin-producing diatom *Skeletonema marinoi* (*** with p value<0.001, students't-test, GraphPad Software).

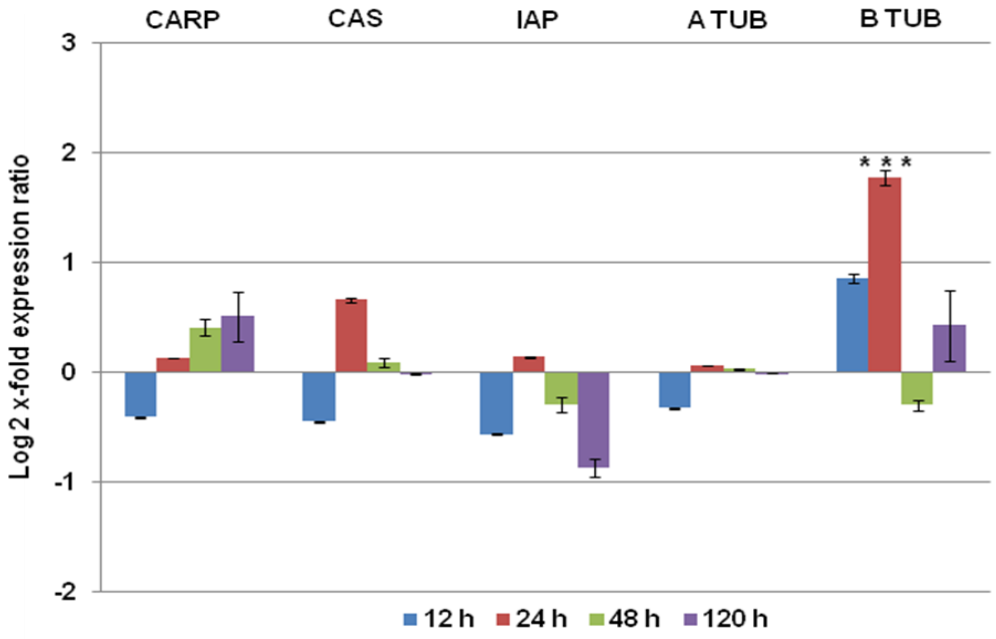

Figure 4.6. Expression levels of genes involved in apoptotis regulation and mitotic spindle formation and stability in Atlantic *Calanus helgolandicus* females fed on the oxylipin-producing diatom *Skeletonema marinoi* (*** with p value<0.001, students't-test, GraphPad Software).

4.3.3 Egg viability

Egg viability for Mediterranean *C. helgolandicus* females fed on *S. marinoi* (SKE) showed a time dependent decrease from 90%, after 48 h, to 40%, after 120 h (Figure 4.7). Egg viability for females fed on the control flagellate *R. baltica* (RHO) remained constant at about 80%. After 72 h,RHO females did not produce eggs.

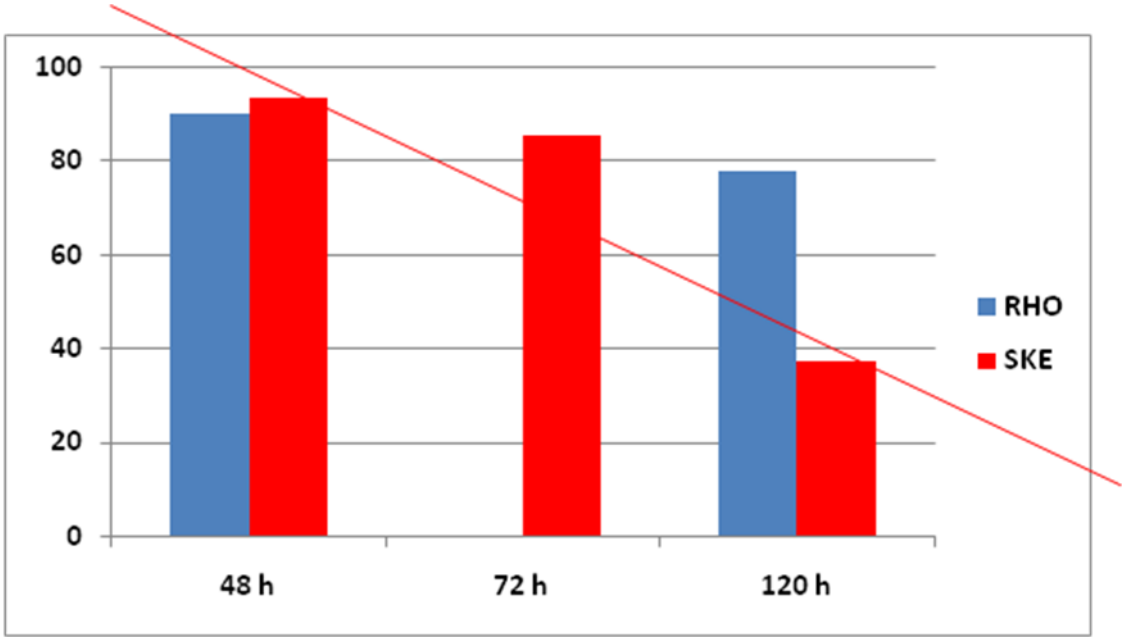

Figure 4.7. Percentage egg viability (y-axis) for Mediterranean *C. helgolandicus* females

By contrast, egg viability for Atlantic *C. helgolandicus* females fed on *S. marinoi* (SKE) showed a time dependent increase from 0 to 80% (Figure 4.8). Egg viability for females fed on the control flagellate *R. baltica* (RHO) was about 80% during the experiment. After 120 h of feeding on RHO females did not produce eggs.

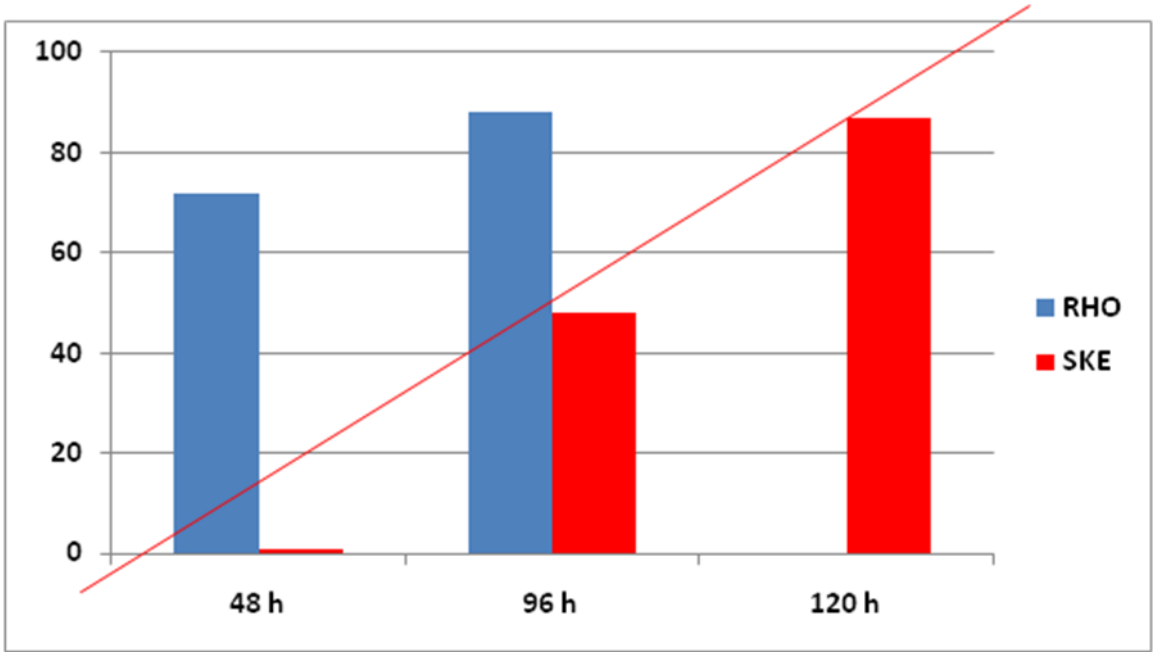

Figure 4.8. Percentage egg viability (y-axis) for Atlantic *C. helgolandicus* females.

4.4 Discussion

Calanus helgolandicus populations collected in the Adriatic Sea and in the North Atlantic Ocean responded differently to the oxylipin-producing diatom *Skeletonema marinoi*. Previous studies have examined biogeographical distributions of *C. helgolandicus* populations (Beaugrand, Helaouet, 2008; Papadopoulos, *et al.*, 2005). Analyses of population genetic parameters of Mediterranean and Atlantic Ocean *C. helgolandicus* populations suggest Pleistocene divergences (Papadopoulos et al., 2005). Our data concord with previous findings of 3 out of 518bp differences in the COI sequences of NE Atlantic and Adriatic Sea *C. helgolandicus* populations confirming that the two populations are very closely related (genetic divergences between 0.22% and 0.57%, Papadopoulos et al., 2005). Congeneric interspecific divergences of COI in calanoid copepods are higher (17% to 22% between *C. finmarchicus, C. helgolandicus*

and *C. glacialis*, (Bucklin, *et al.*, 1999); 7–25% between ten *Calanus* species (Hill, *et al.*, 2001). These results show that divergences between Mediterranean and Atlantic populations are remarkably lower than inter-species differences and that the expression patterns may depend on the possible adaptation of the populations to local environmental conditions. Similar studies on genetic divergence between Atlantic and Mediterranean populations have been obtained for marine fishes (Bargelloni, *et al.*, 2003) and a chaetognath (Peijnenburg, *et al.*, 2004). To our knowledge, this is the first study to compare gene expression patterns between *C. helgolandicus* populations.

Both Atlantic and Mediterranean populations showed a down-expression of ALDH3 and HSP70, and the up-regulation of the detoxification enzyme GST after 12 h of ingestion of *S. marinoi*, even if it was more pronounced for the Atlantic population. GST did not show significant changes after 12 h and 24 h of exposure to water accommodated fractions (WAF) of oil (Hansen, *et al.*, 2011) and diethanolamine (DEA) (Hansen, *et al.*, 2010) in the copepod *Calanus finmarchicus* (*C. finmarchicus*), while an increase in GST was found after 12 h of Naphthalene exposure (Hansen, *et al.*, 2008b). GST levels were reduced after 10, 20, 30 and 60 min of exposure to 2 mM H_2O_2 and increased after 6, 12 and 24 h Cu and Mn heavy metal treatment (Lee, *et al.*, 2007) in the copepod *Tigriopus japonicus*.

On the other hand, enzymes involved in free radical detoxification, CAT and SOD, were up-regulated after 12 h of exposure to a diatom diet in the Atlantic population of *C. helgolandicus*, but not in the Mediterranean population These data suggest an immediate specific capability of the Atlantic population to protect itself against radical toxicants, probably because this population is more frequently exposed to diatom blooms (Poulet, *et al.*, 2006) and may have evolved mechanisms to cope with deleterious diatom metabolites. After 24 h of feeding on *S. marinoi* CAT was up-

regulated as well. A simultaneous increase in both SOD and CAT has also been observed in *C. finamrchicus* after 12 h of exposure to low naphthalene concentrations (Hansen, *et al.*, 2008b). Thereafter levels for both antioxidants returned to basal levels, except after 48 h, when CAT levels were still elevated in copepods exposed to intermediate naphthalene concentrations. Whereas Hansen and co-workers (2008) concluded that there was no clear relationship between antioxidant mRNA levels and exposure time/concentration our data suggest that antioxidant defence (e.g. GST, SOD and CAT) genes may be activated soon after stress exposure (i.e. 12 h), while a more specific detoxification system could be activated somewhat later (24 h).

In fact, three ALDHs (ALDH8, 7 and 6) increased after 24 h in the Atlantic population compared to 5 days of feeding on *S. marinoi* in the Mediterranean population. In particular, a 3-fold increase was observed for ALDH7 and 2-fold for ALDH2, while ALDH9 and ALDH3 increased by a little bit more than 1-fold. To our knowledge, ALDHs have not been studied in other copepod species. ALDHs are involved in the detoxification of endogenous and exogenous aldehydes in humans, including aliphatic, fatty, amine and aromatic aldehydes that are dangerous for human health. Mutations and polimorphisms in ALDH genes, leading to loss of function, are associated with various deseases, such as alcohol-related diseases, Sjögren-Larsson syndrome (SLS) and cancer (Marchitti, *et al.*, 2008). Aldehydes in fragrances are one of the most common causes of allergic contact dermatitis (O'Brien, *et al.*, 2005), and elevated aldehydes are often associated with the pathogenesis of Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, cerebral ischemia, cataracts, and pathologic corneas diseases (Estey, *et al.*, 2007; Marchitti, *et al.*, 2008; Ou, *et al.*, 2002; Vasiliou, *et al.*, 2004). To our knowledge, ALDHs have been studied in few marine organisms: members of the ALDH gene superfamily have been identified in the marine

picoeukaryote *Ostreococcus tauri* and the unicellular green algae *Chlamydomonas reinhardtii* (Wood, Duff, 2009).

Of the 18 transcripts analyzed by RT-qPCR at least 50% were strongly down-regulated (aldehyde dehydrogenase 9, 8 and 6, cellular apoptosis susceptibility and inhibitor of apoptosis IAP proteins, heat shock protein 40, alpha- and beta-tubulins) after 48 h in the Mediterranean population compared to the control. For the Atlantic population, only GST and CYP were down-expressed, whereas other genes showed no significant changes in expression profiles.

Previous studies have already shown that egg viability was reduced for copepod females fed on the oxylipin producing diatom *S. marinoi* compared to the control (Ianora, *et al.*, 2004; Miralto, *et al.*, 1999). Our data on the Mediterranean population confirm that egg viability shows a time dependent decrease the longer it feeds on a diatom diet (Figure 7). By contrast, the Atlantic *C. helgolandicus* population showed a completely different pattern, with a time dependent increase in egg viability of females fed on the same diatom diet (Fugure 8). Does this denote that the Atlantic population is less susceptible to diatom toxins compared to the Mediterranean population?

Future studies on both *C. helgolandicus* populations may provide insight on the maximum PUA concentrations that copepods can tolerate and the minimum quantity of ingested algae necessary to induce a stress response in *C. helgolandicus* females. Our results suggest that there are population-related responses of copepods exposed to the toxic diatom *S. marinoi* thereby differentially impacting local food webs and economically important fisheries.

Chapter 5:

Calanus helgolandicus expression levels during natural diatom blooms in the North Adriatic Sea

In preparation as:

Lauritano Chiara, Carotenuto Ylenia, Procaccini Gabriele, Miralto Antonio and Ianora Adrianna.
Calanus helgolandicus expression levels during natural diatom blooms in the North Adriatic Sea.
In preparation.

5.1 Brief introduction

Strong diatom blooms that negatively impact the hatching success of important zooplankton grazers such as calanoid copepods have been described as occurring in late winter in the Northern Adriatic Sea (Miralto et al. 1999). These authors were the first to identify low molecular weight polyunsaturated aldehydes (PUAs) from diatoms and to show that only 12% of copepod eggs hatched during a bloom of the diatom *Skeletonema marinoi* in the North Adriatic Sea in 1997 and 1998 compared to 90% in post-bloom conditions. Successively Ianora et al. (2004) showed that nauplii collected from wild *Calanus helgolandicus* females during the *S. marinoi* bloom from February to May 2003, did not survive to adulthood when reared on a diet of this diatom. Individuals died at or before the Copepodite II stage, except on the 13 and 27 March, when some nauplii (20%) reached adulthood. Survivorship improved when copepods were fed on the control non-diatom *Prorocentrum minimum* but few individuals reached adulthood except after late April when the *S. marinoi* bloom had collapsed and had been replaced by a mixed diatom-flagellate microbial assemblage. Percentage egg viability during the bloom was low (16.8 to 39.7%), as already showed in earlier results (Miralto et al. 1999). Thus, maternal diatom diets in the field negatively affected not only egg hatching success, but also the development of nauplii that hatched but could not survive to adulthood.

In order to better investigate the negative effects of natural diatom diets on copepods, in this part of my thesis I analyzed expression levels of the genes of interest (involved in generic stress response, phase I and phase II metabolic reactions, aldehyde detoxification and apoptosis regulation) in *C. helgolandicus* specimens collected during two *S. marinoi* blooms in 2010 and 2011 in order to compare these results with those

obtained in the laboratory where *C. hegolandicus* females were fed laboratory cultures of *S. marinoi* (Chapters 2, 3 and 4).

5.2 Methods

5.2.1 Copepod sampling

Calanus helgolandicus specimens were collected in the North Adriatic Sea during the winter *S. marinoi* blooms in 2010 and 2011. In 2010, samples were collected in April, May and June, while in 2011 samples were collected in March (15/3/11 and 30/03/11) and April. Zooplankton was collected from the bottom to the surface with a Nansen net (200 micron mesh size) (Figure 5.1) at four stations: station 2 (ST2), far from the coast, station 3 (ST3), station 4 (ST4) and station 5 (ST5), near the coast (Table 1 reports sampling sates, analyzed stations and coordinate sites). Triplicate samples of about 5 adult female *C. helgolandicus* specimens were collected at each station and transferred to 1L bottles filled with 0.22 μm filtered sea water (FSW). Females were kept at about 20°C for 24 h to eliminate any algal residues in the gut. Each replicate was then carefully transferred to 500 μl Trizol Reagent (Invitrogen), frozen directly in liquid nitrogen until arrival in Naples where they were stored at -80°C until RNA extraction. Phytoplankton concentration analyses are not available yet.

Figure 5.1. Nansen net used for the samplings.

Station	Date	Latitude	Longitude
ST2	21/04/2010	44°55’,824	12°53’,104
ST3	21/04/2010	44°55’,885	12°49’,430
ST4	21/04/2010	44°55’,990	12°45’,734
ST5	21/04/2010	44°56’,010	12°42’,032
ST2	25/05/2010	44°55’,824	12°53’,104
ST3	25/05/2010	44°55’,885	12°49’,430
ST4	25/05/2010	44°55’,990	12°45’,734
ST5	25/05/2010	44°56’,010	12°42’,032
ST2	09/06/2010	44°55’,824	12°53’,104
ST3	09/06/2010	44°55’,885	12°49’,430
ST4	09/06/2010	44°55’,990	12°45’,734
ST5	09/06/2010	44°56’,010	12°42’,032
ST2	15/03/2011	44°55’,824	12°53’,104
ST3	15/03/2011	44°55’,885	12°49’,430
ST4	15/03/2011	44°55’,990	12°45’,734
ST5	15/03/2011	44°56’,010	12°42’,032
ST2	30/03/2011	44°55’,824	12°53’,104
ST3	30/03/2011	44°55’,885	12°49’,430
ST4	30/03/2011	44°55’,990	12°45’,734
ST5	30/03/2011	44°56’,010	12°42’,032
ST2	09/04/2011	44°55’,835	12°53’,119
ST3	09/04/2011	44°55’,903	12°49’,480
ST4	09/04/2011	44°55’,985	12°45’,760
ST5	09/04/2011	44°56’,037	12°42’,053

Table 5.1. This table reports the station where zooplankton samples were collected, sampling dates and site coordinates.

5.2.2 Egg viability

At each station, 10 *C. helgolandicus* adult females were sorted from zooplankton samples and incubated in 50 ml bottles with sea water containing a natural phytoplankton assemblage. After 24 h females were removed and eggs counted. The number of hatched eggs was determined after an additional 24 h.

5.2.3 RNA extraction and cDNA synthesis

Total RNA was extracted using a modified Trizol manufacturer's protocol (Invitrogen) (For more details see Appendix 1). RNA quantity and purity was assured by Nano-Drop (ND-1000 UV-Vis spectrophotometer; NanoDrop Technologies), RNA quality by gel electrophoresis. 1 μg of each RNA was retrotrascribed 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, 1μl 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.

5.2.4 Reverse transcription-Quantitative real time polymerase chain reaction (RT-qPCR)

The fluorescent dye SYBR GREEN was used to evaluate expression levels of the selected genes by RT-qPCR. Fluorescence was monitored once per cycle after product extension and increased above background fluorescence at a cycle number that depended on the initial template concentration (For protocol details see APPENDIX 2).

To study expression levels for each target gene relative to the most stable RG, S20 (as discussed in chapter 2, Lauritano *et al*., 2011a), we used the REST tool (Relative expression software tool) (Pfaffl, et al., 2002). Statistical analysis was performed using GraphPad Prim statistic software, V4.00 (GraphPad Software).

5.3 Results

5.3.1 2010: Egg viability and Gene expression analyses

Egg viability was assessed for each station during the course of three sampling occasions in April, May and June 2010. In April, egg viability ranged from 44% at station 5 to 69% at station 3, while in May egg viability was strongly reduced (from 23% at station 3 to 26% at station 4) (data are mean percentage egg viability for 10 females ± SE) (Figure 5.1). During post-bloom conditions in June egg viability was very high, between 91% at station 2 and 99% at station 3. Thus animals collected in June were considered as controls during the successive gene expression analyses.

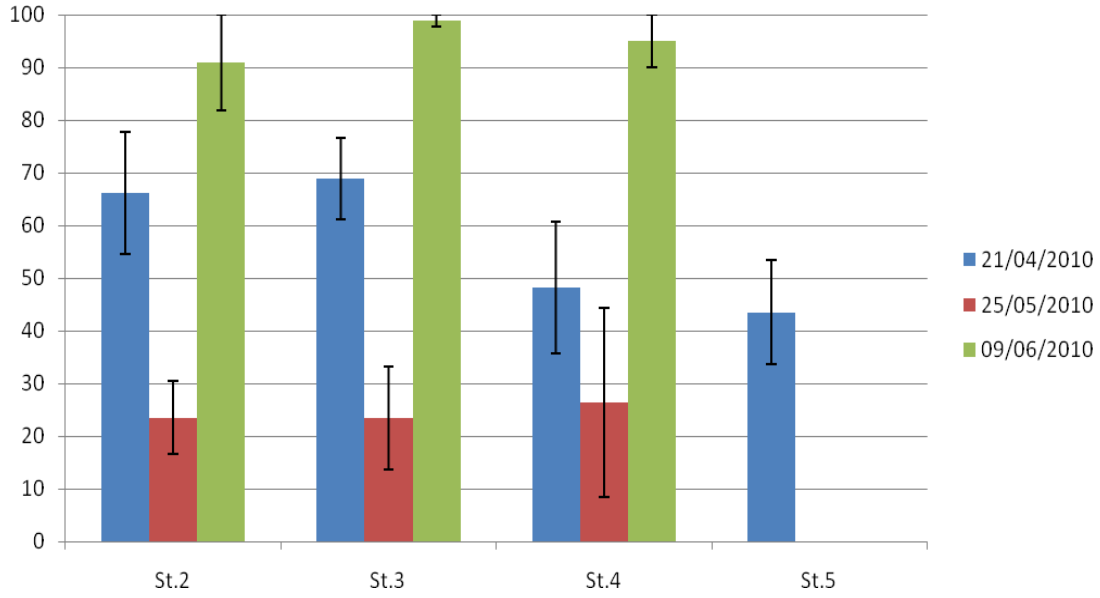

Figure 5.1. Egg viability (percentage of egg viability on y-axis) for *Calanus helgolandicus* females collected in the North Adriatic Sea during a natural diatom bloom in 2010. Sampling dates were 21/04/2010, 25/05/2010 and 09/06/2010. Data are expressed as mean percentage egg viability for 10 females ± SE.

Of the genes involved in generic stress response, HSP70 and GST were both significantly up-regulated in both April and May (p value<0.01, for HSP70 for both

months, p value<0.05, for GST for both months, students't-test, GraphPad Software). In May, HSP70 and GST expression levels were higher than in April with a 2-fold up-regulation of both genes. By contrast, CYP4, CAT and SOD expression levels were significantly reduced in both months (p value<0.001, for all three genes, students't-test, GraphPad Software) (Figure 5.2).

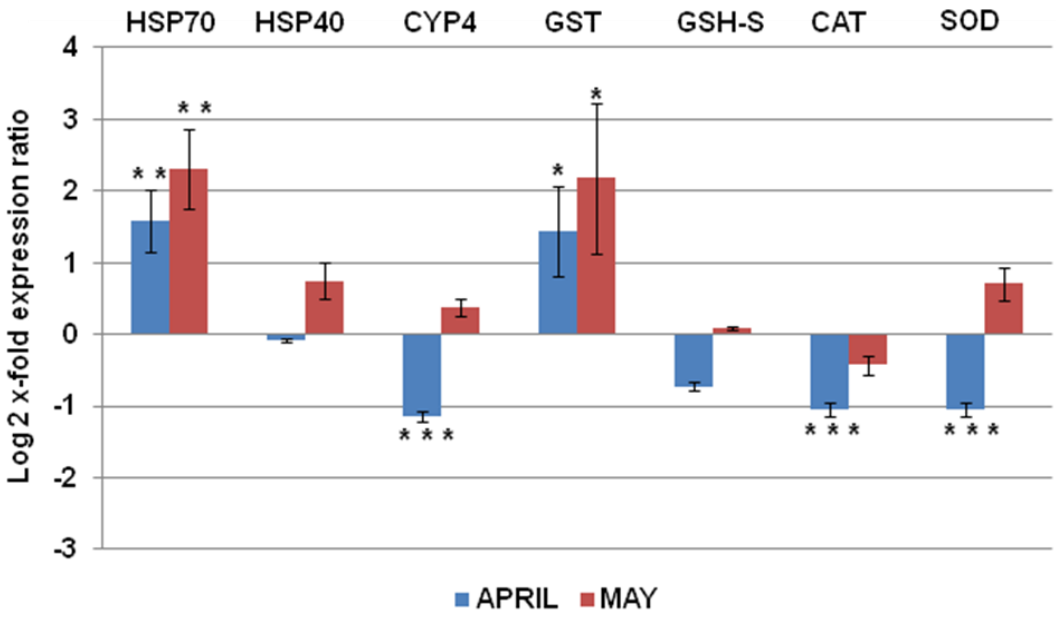

Figure 5.2. Stress response and defense system expression patterns in *Calanus helgolandicus* females collected during a natural bloom of the diatom *Skeletonema marinoi* in 2010 (* with p value<0.05, ** with p value<0.01, *** with p value<0.001, students't-test, GraphPad Software).

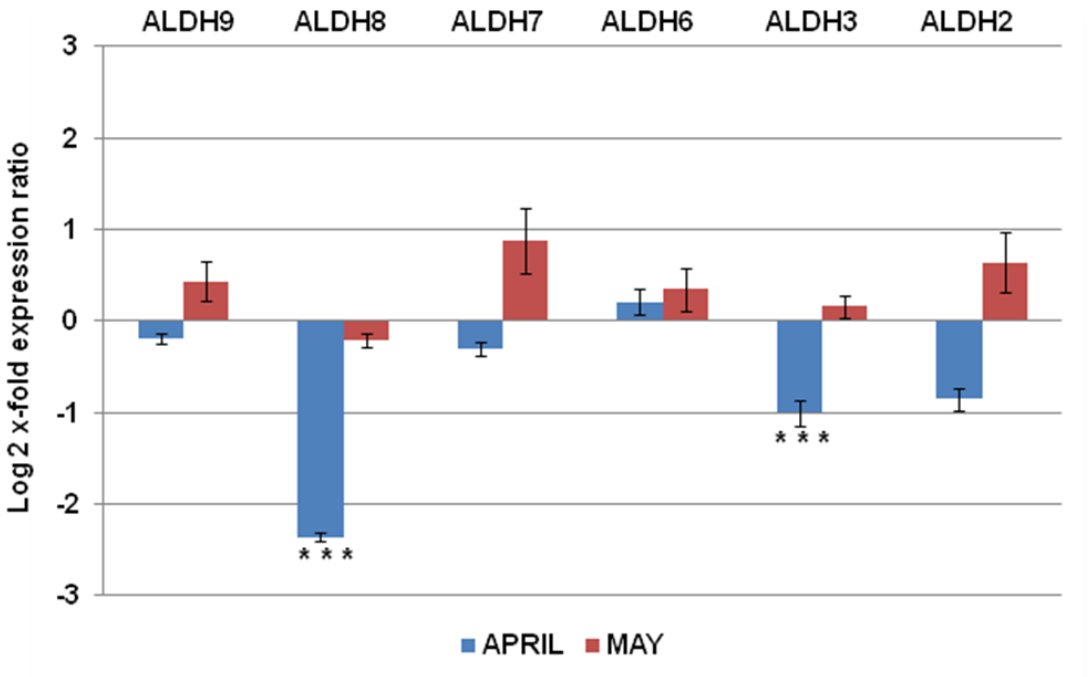

Figure 5.3. Aldehyde dehydrogenase expression levels in *Calanus helgolandicus* females collected during a natural bloom of the diatom *Skeletonema marinoi* in 2010(*** with p value<0.001, students't-test, GraphPad Software).

Of the analyzed aldehyde detoxification enzymes, ALDH2, ALDH3 and ALDH8 expression levels were down-expressed. ALDH3 and ALDH8 were significantly reduced (p value<0.001, for both genes, students't-test, GraphPad Software) (Figure 5.4). CARP, CAS and alpha TUB did not show significant changes, while IAP expression levels significantly decreased in April (p value<0.001, students't-test, GraphPad Software) and BTUB was significantly 2-fold up-regulated in May (p value<0.05, students't-test, GraphPad Software) (Figure 5.5).

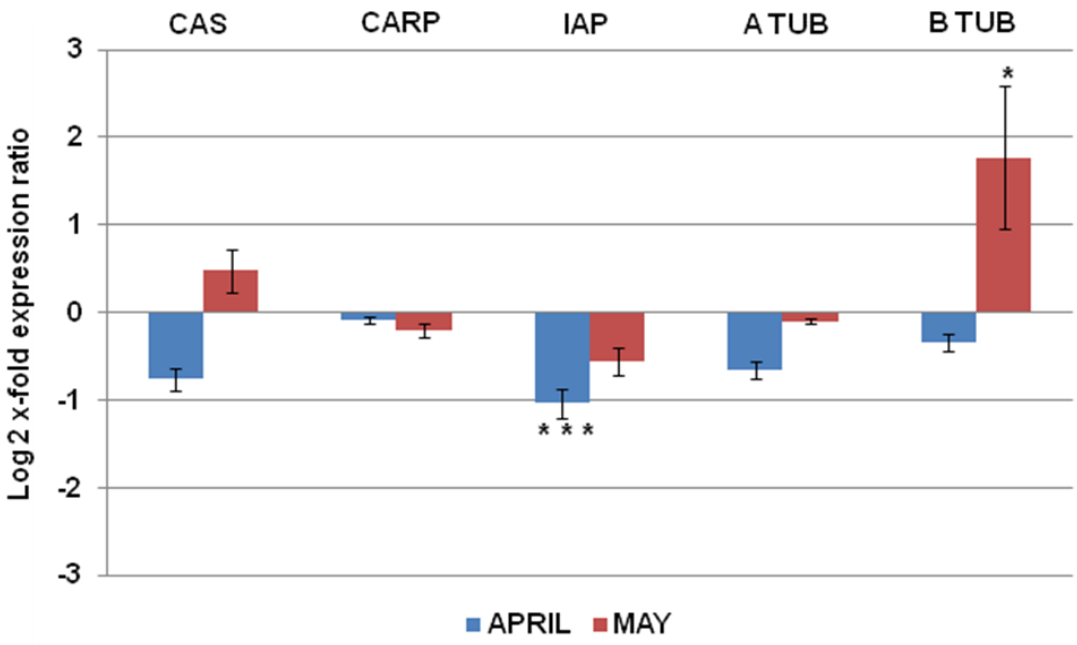

Figure 5.4. Expression levels of genes involved in apoptotis regulation and mitotic spindle formation in *Calanus helgolandicus* females collected during a natural bloom of the diatom *Skeletonema marinoi* in 2010 (* with p value<0.05, *** with p value<0.001, students't-test, GraphPad Software).

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5.3.2 2011: Egg viability and Gene expression analyses

Egg viability was higher on 15/03/2011 compared to later sampling periods (from 71 to 92%) (Figure 5.5). By 29/03/2011 egg viability had decreased, ranging from 61% at station 5 to 93% at station 2. By 09/04/2011 egg viability had increased again and was between 69% to 87% (data are mean percentage egg viability for 10 females ± SE). Stations with the lowest egg viability on each of the three sampling occasions were analyzed by RT-qPCR. For the molecular analyses, station 3 (15/03/2011) with the highest egg viability (>90%) was used as a control.

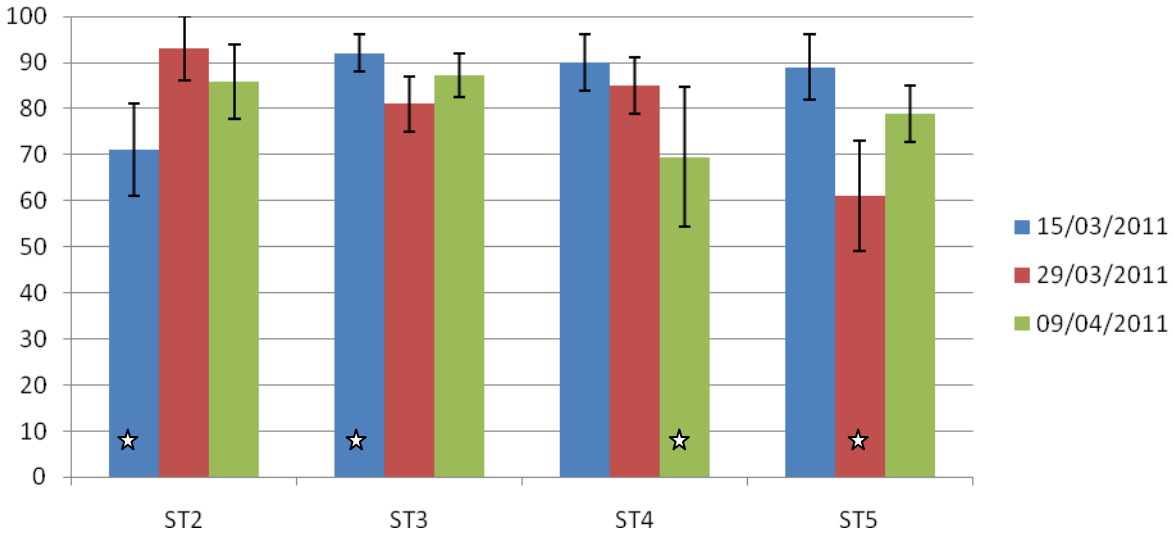

Figure 5.5. Egg viability (percentage of egg viability on y-axis) for *Calanus helgolandicus* females collected in the Adriatic Sea during a natural diatom bloom in 2011. Sampling dates were 15/03/2011, 30/03/2011 and 09/04/2011. Data are expressed as mean for % egg viability for 10 females ± SE. Stations analyzed by RT-qPCR are indicated by white stars.

Copepods collected in 2011 showed altered patterns in defense and stress response systems. HSPs were up-regulated, even if this increase was significant only for HSP70 at ST5 29/3/11 (p value<0.05, students't-test, GraphPad Software) (Figure 5.5). Up-regulation was pronounced in copepod females collected at ST4 and ST5. Free radical detoxification systems also increased: SOD was almost 2-fold up-regulated at ST4 and ST5 (p value<0.01 for SOD at ST4, students't-test, GraphPad Software) and GSH-S increased at ST2. On the contrary, CYP mRNA expression levels were reduced, particularly at ST2 and ST5 (p value<0.001 for both stations, students't-test, GraphPad Software). No clear response was associated to the phase II detoxification enzyme GST. GST increased at ST5, decreased at ST4 (p value<0.001, students't-test, GraphPad Software), while at ST2 its expression levels were comparable to the control (Figure 5.6).

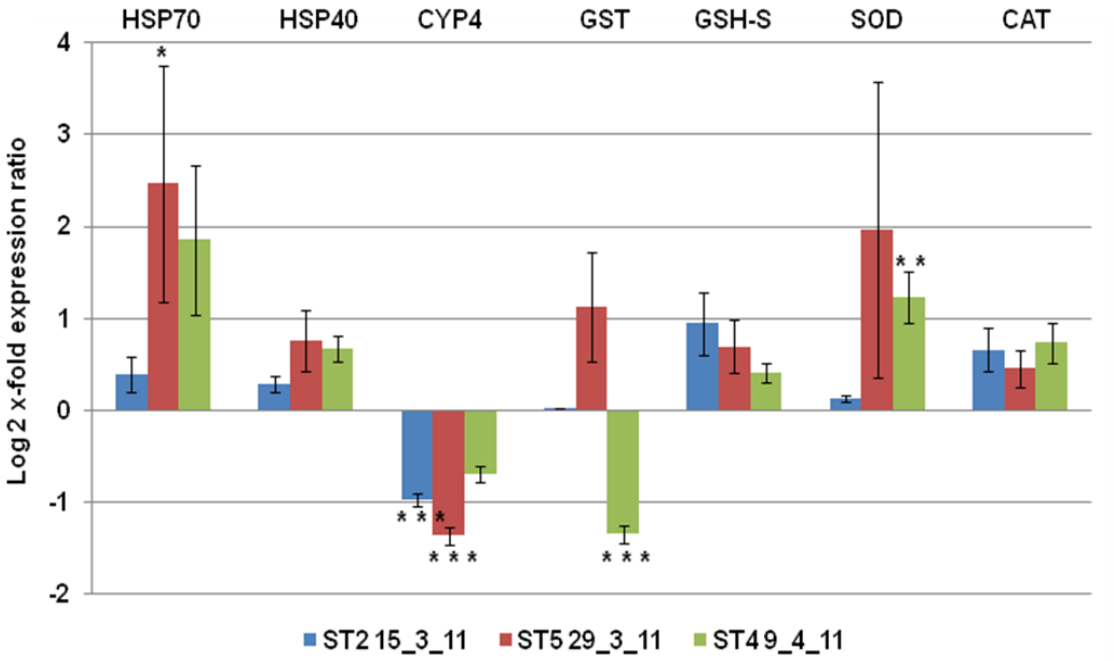

Figure 5.6. Stress response and defense system expression patterns in *Calanus helgolandicus* females collected during a natural bloom of the diatom *Skeletonema marinoi* in 2011 (* with p value<0.05, ** with p value<0.01 and *** with p value<0.001, students't-test, GraphPad Software).

Aldehyde detoxification enzymes did not show particular changes, except for ALDH7 mRNA levels that increased at all three stations (Figure 5.7). There were no apoptotic or anti-apoptotic signals. CARP, CAS, IAP and ATUB mRNA levels, in fact, did not show significant changes while the microtubule subunit BTUB was significantly down-regulated at ST2 and ST4 (p value<0.001, for both genes, students't-test, GraphPad Software). (Figure 5.8).

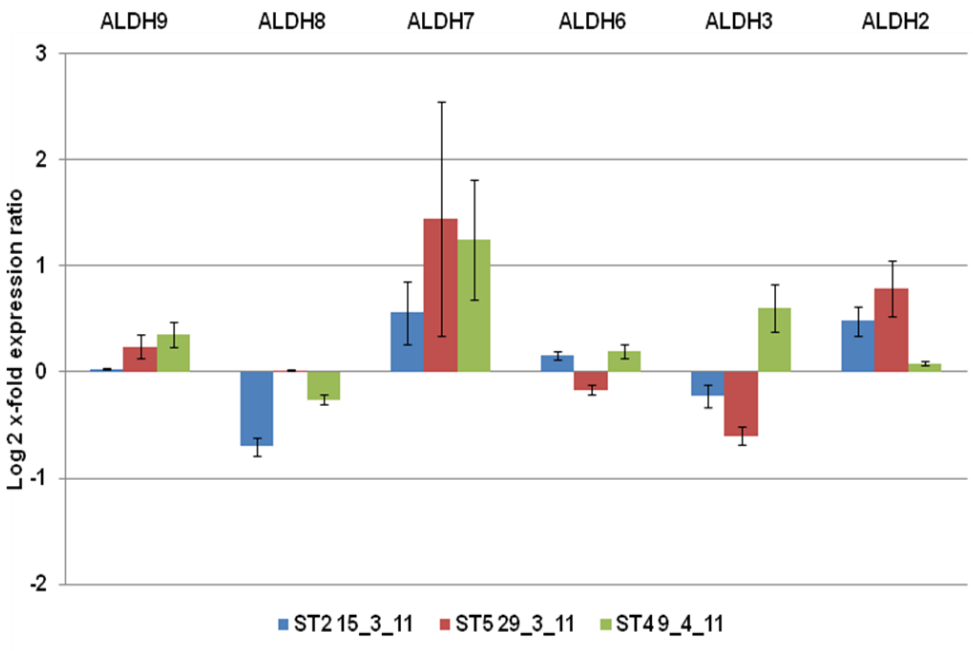

Figure 5.7. Aldehyde dehydrogenase expression levels in *Calanus helgolandicus* females collected during a natural bloom of the diatom *Skeletonema marinoi* in 2011.

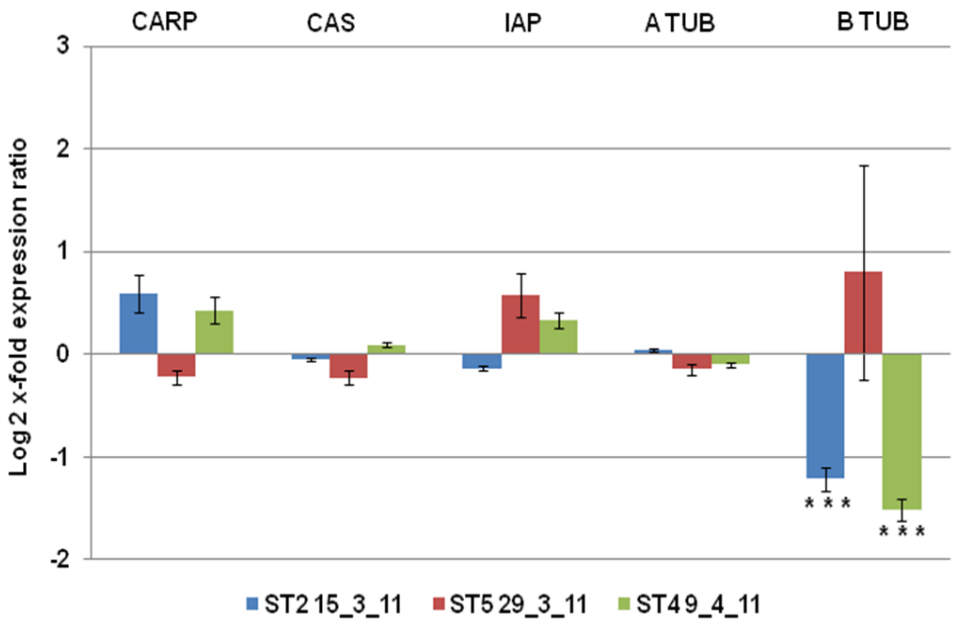

Figure 5.8. Expression levels of genes involved in apoptotis regulation and mitotic spindle formation in *Calanus helgolandicus* females collected during a natural bloom of the diatom *Skeletonema marinoi* in 2011(*** with p value<0.001, students't-test, GraphPad Software).

5.4 Discussion

The highest percentages of hatched *Calanus helgolandicus* eggs were found at the beginning of the *Skeletonema marinoi* diatom bloom in March 2011, and at the end of the bloom (June of the previous year). In April and 2010, egg viability crashed reaching lowest levels of 20% hatching success. In 2011, egg viability was also high at the beginning (from 71 to 92%) and at the end (from 81 to 93%), whereas it decreased in April (from 69 to 87%).

We used females collected in June during post-bloom conditions (see Miralto et al. 1999) as control for RT-qPCR analyses of samples collected in 2010. In 2011 it was not possible to collect females in June; hence we used as a control females collected at the station where highest egg viability occurred (ST 3, 15/03/2011).

Interestingly, in both years, expression levels of the chaperoning protein HSP70 were up-regulated in females collected at stations with lowest egg viability (April and May 2010, and ST5 of the 30/03/2011). HSPs are known to be activated in response to various environmental stress factors such as heat, hypoxia, UV radiation, and chemical exposure (Bierkens, 2000; Feder, Hofmann, 1999; Tartarotti, Torres, 2009) and can be involved in protein folding and unfolding, and degradation of mis-folded or aggregated proteins (Sorensen, *et al.*, 2003). Romano et al. (Romano, *et al.*, 2011) have recently shown that sea urchins activate HSP70 when challenged with low concentrations (0.25 µg/ml) of the PUA decadienal thereby protecting embryos against the toxic effects of this aldehyde. By contrast, *C. helgolandicus* females fed on the oxylipin-producing diatom *S. marinoi* during laboratory feeding experiments did not show HSP70 up-regulation (Lauritano, *et al.*, 2011b) (Chapter 3). Hence our new data suggest that the protective role of HSP70 is expressed only at sea and not in the laboratory. Possibly this

may be due to strain-specific differences in oxylipin content of *S. marinoi* (Gerecht, *et al.*, 2011) or to dilution effects resulting from mixed diets (Turner, *et al.*, 2000) in the field. During field experiments the phase II detoxification enzyme GST followed the same pattern as HSP70. GST was, in fact, up-regulated in females collected at stations with lowest egg viability (April and May in 2010, and ST5 on 30/03/2011). In previous laboratory experiments, GST expression patterns were unaffected when *C. helgolandicus* females were fed on *S. marinoi* (Lauritano, *et al.*, 2011b). Kozlowsky-Suzuki and co-workers (2008) suggested that GST enzymatic activity did not seem to play a role in detoxification of copepods exposed to toxic dinoflagellate algae such as *Alexandrium minutum* and *Alexandrium tamarense*, which contained Paralytic Shellfish Poisoning (PSP) toxins, and the dinoflagellate *Prorocentrum lima* with Diarrhetic Shellfish Poisoning (DSP) toxins. Possibly GST expression levels may only be up-regulated in field conditions since both in our case (Lauritano, *et al.*, 2011b) and in the case of Kozlowsky-Suzuki (2008) this enzyme was not activated in laboratory conditions. Interestingly, BTUB expression levels were affected in both years at sea, even if in different ways. BTUB was up-regulated in copepods collected in May 2010, while it was down-regulated in animals collected at station 2 (15/03/2011) and station 4 (09/04/2011). This opposite response did not correlate with egg production. This opposing pattern of up- and down-regulation suggests that in 2010 females were less affected by the bloom compared to 2011. BTUB levels were strongly down-regulated in *C. helgolandicus* females fed *S. marinoi* during laboratory feeding experiments (Lauritano, *et al.*, 2011a)(Chapter 2).

Phytoplankton concentrations are not available yet, but during bloom conditions *S. marinoi* can reach 10^9 cell/ml (Bastianini personal communication), comparable with the concentrations used in laboratory experiments.

Gene expression changes are an important early signal to indicate stress conditions compared to other physiological parameters and/or phenotype changes. Future studies on gene expression levels in *C. helgolandicus* specimens during natural diatom blooms in the Adriatic Sea, with more samplings during the same bloom, and with simultaneous measurements of phytoplankton composition and diatom chemical analyses may help identify the oxylipin concentrations in natural diatom blooms that trigger a response in copepod genes involved in detoxification systems or apoptosis regulation.

Chapter 6:

Gene expression patterns in the copepod *Calanus helgolandicus* fed the toxic dinoflagellate *Karenia brevis*.

In preparation as:

Lauritano Chiara, Carotenuto Ylenia, Procaccini Gabriele, Miralto Antonio and Ianora Adrianna.
Calanus helgolandicus exposed to the toxic dinoflagellate *Karenia brevis*.
In preparation.

6.1 Brief introduction

Karenia brevis (*K. brevis*), also known as *Gymnodinium breve*, is a microscopic, single-celled and photosynthetic organism common in the Gulf of Mexico waters and the mid and south Atlantic coast of the USA (Landsberg, 2002). This dinoflagellate naturally produces potent neurotoxins, termed brevetoxins. Brevetoxins are polycyclic ethers that, by binding to specific sites on the voltage-sensitive sodium channel, alter membrane properties of excitable cells by shifting activation to more negative potentials and inducing membrane depolarization (Landsberg, 2002; Naar, *et al.*, 2007; Pierce, Henry, 2008). These molecules are principally associated with neurotoxic shellfish poisoning (NSP) and harmful algal blooms (HABs). HABs can have serious economic consequences for aquaculture and beach tourism. At times, coastal marine species "bloom" by reproducing in such great numbers that the water may appear golden or red, producing a "red tide" (Landsberg, 2002). During *K. brevis* blooms, massive fish kills can occur, as well as high mortality of marine mammals, sea turtles, sea birds and benthic communities. Public health effects due to shellfish contamination or inhalation of air-borne toxins have been reported as well (Pierce, Henry, 2008; Watkins, *et al.*, 2008). Mussels, clams and oyster, in fact, feeding on these microscopic algae accumulate their neurotoxins and toxic algal metabolites. In addition to direct impacts, the decomposing biomass adds stress to the environment by depleting oxygen from the water, affecting benthic organisms, such as seagrass and patch reef communities that require years for recovery (Pierce, Henry, 2008).

It is often assumed that harmful algal toxins are grazing deterrents to discourage zooplankton grazers from consuming these algae. Some laboratory studies have suggested that toxic algae are either not consumed by various grazers or that grazers ingesting toxic algae suffer adverse effects such as reduced feeding rates, diminished

reproductive success, behavioral modification or increased mortality (Cohen, *et al.*, 2007; Kubanek, *et al.*, 2007; Prince, *et al.*, 2006). In the case of *Karenia brevis*, copepod swimming and photobehavior, both behaviors of which are involved in predator avoidance, were impaired at sublethal *K. brevis* and PbTx-2 levels (Cohen et al., 2007). Significant mortality was only observed in one (*Temora turbinate*) of the three copepod species tested at the highest test concentration (1×10^7). Egg production rates and egg viability for Mediterranean *Calanus helgolandicus* females fed on *K. brevis* declined steadily over time, reaching almost zero levels after 4days of feeding on this alga (Turner et al., in preparation).

In order to better understand the effects of *K. brevis* on *C. helgolandicus* at the molecular level, we conducted laboratory feeding experiments using *K. brevis*, Wilson clone, isolated in 1953 (Lekan and Tomas, 2010) and the Mediterranean copepod *Calanus helgolandicus*. Expression levels of selected genes of interest were analyzed in copepod females fed for three days on *K. brevis*.

6.2 Methods

6.2.1 Copepod sampling and Feeding Experiments

Adult female *Calanus helgolandicus* specimens were sampled from a 500 L re-circulating copepod breeding system (Buttino et al., 2011, Carotenuto, Esposito, Pisano, Lauritano et al., 2011), isolated under a Leica stereomicroscope and transferred to 1 L bottles filled with 0.22 μm filtered sea water (FSW) at about 20°C. Triplicate bottles containing about 20 adult females were fed *ad libitum* either unialgal diets of the control non-oxylipin producing dinoflagellate *Prorocentrum minimum* (*P. minimum*) (5000 cells/ml) or the toxin-producing dinoflagellate *Karenia brevis* (*K. brevis*) (3400

cells/ml). The neurotoxic dinoflagellate *K. brevis* was the Wilson clone, isolated in 1953 (Lekan, Tomas, 2010) obtained in 2008 from Patricia Tester, National Ocean Service, NOAA, Beaufort, North Carolina, USA. The *P. minimum* strain belongs to the Stazione Zoologica Anton Dohrn culture collection. *P. minimum* and *K. brevis* were cultured in glass jars with 0.22 μm-filtered FSW enriched with k medium at 20^{o}C and on a 12:12 hr dark:light cycle. Every day FSW was replaced in each bottle and new food added at the same concentration as before. After 3 days of feeding, triplicate sub-samples of 5 animals for each diet were collected and transferred to FSW for 24h to eliminate any algal residues in the gut. After this, each replicate was carefully transferred to 500 μl Trizol Reagent (Invitrogen), frozen directly in liquid nitrogen and stored at -80°C until RNA extraction.

6.2.2 RNA extraction and cDNA synthesis

Total RNA was extracted using a modified Trizol manufacturer's protocol (Invitrogen) (For more details see Appendix 1). RNA quantity and purity was tested by Nano-Drop (ND-1000 UV-Vis spectrophotometer; NanoDrop Technologies), RNA quality by gel electrophoresis. 1 μg 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, 1μl 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 at 85°C for 5 min.

6.2.3 Reverse transcription-Quantitative real time polymerase chain reaction (RT-qPCR)

The fluorescent dye SYBR GREEN was used to evaluate expression levels of the selected genes by RT-qPCR. Fluorescence was monitored once per cycle after product extension and increased above background fluorescence at a cycle number that depended on the initial template concentration. (For protocol details see APPENDIX 2).

In order to analyze expression levels of specific genes of interest (GOI), a panel of putative reference genes (RGs) was first screened to find the most stable genes and best RGs in the new experimental conditions. 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).

Then expression level analyses of specific GOIs were performed. Putative RGs were elongation factor 1α (EFA), adenosine 3-phosphate synthase (ATPs), histone 3 (HIST), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal units (18S, S7, S20), ubiquitin (UBI), β-actin (ACT), and alpha- and beta-tubulins (ATUB and BTUB, respectively); GOIs were six Aldehyde dehydrogenases (ALDH2, ALDH3, ALDH6, ALDH7, ALDH8, ALDH9), Cytochrome P450-4 (CYP4), Catalase (CAT), Superoxide Dismutase (SOD), Glutathione S-Transferase (GST), Glutathione Synthase (GSH-S), Inhibitor of Apoptosis Protein (IAP), Cell Cycle and Apoptosis Regulatory 1 Protein (CARP), Cellular Apoptosis Susceptibility Protein (CAS) and alpha-tubulin (ATUB).

To study expression levels for each target gene relative to the most stable RGs (S20, S7, GAPDH and BTUB), we used the REST tool (Relative expression software tool) (Pfaffl, et al., 2002). Females fed for three days on the control dinoflagellate *Prorocentrum minimum* were used as control. Statistical analysis was performed using GraphPad Prim statistic software, V4.00 (GraphPad Software).

6.2.4 Microscopy

Images of newly spawned eggs were acquired for image analysis for females fed on the control or tested diets; acquisition of images was performed until egg hatching using a Zeiss-LSM 510 META confocal microscope (Laser 488, emission filter: BP 500–550).

6.3 Results

6.3.1 Validation of best reference genes for RT-qPCR

Raw Ct data of potential RGs are reported in Figure 6.1. According to the mathematical approach of BestKeeper, RG expression stability considers the standard deviation of the Ct values (Figure 6.2). Hence the most stable RGs have a standard deviation (SD) lower than 1 and these included BTUB, GAPDH, S7, IST, EFA, UBI, ATPS, A TUB and S20. BestKeeper analysis (Pfaffl, et al., 2004) indicated BTUB as the most stable gene, followed by GAPDH and S7.

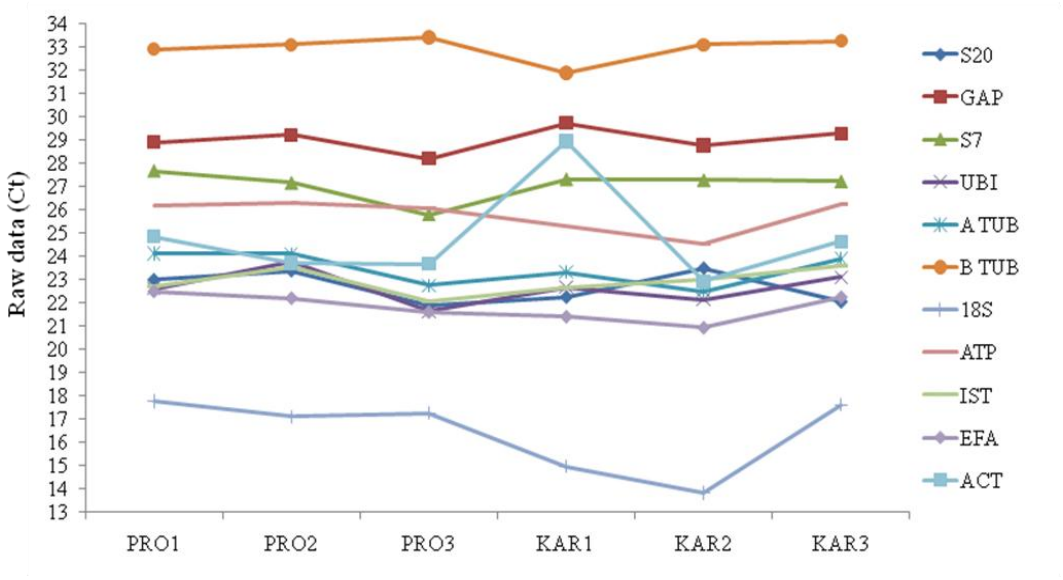

Figure 6.1. RT-qPCR raw data of putative reference genes. Putative RGs were elongation factor 1α (EFA), adenosine 3-phosphate synthase (ATPs), histone 3 (HIST), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal units (18S, S7, S20), ubiquitin (UBI), β-actin (ACT), alpha- and beta-tubulins (ATUB and BTUB, respectively)

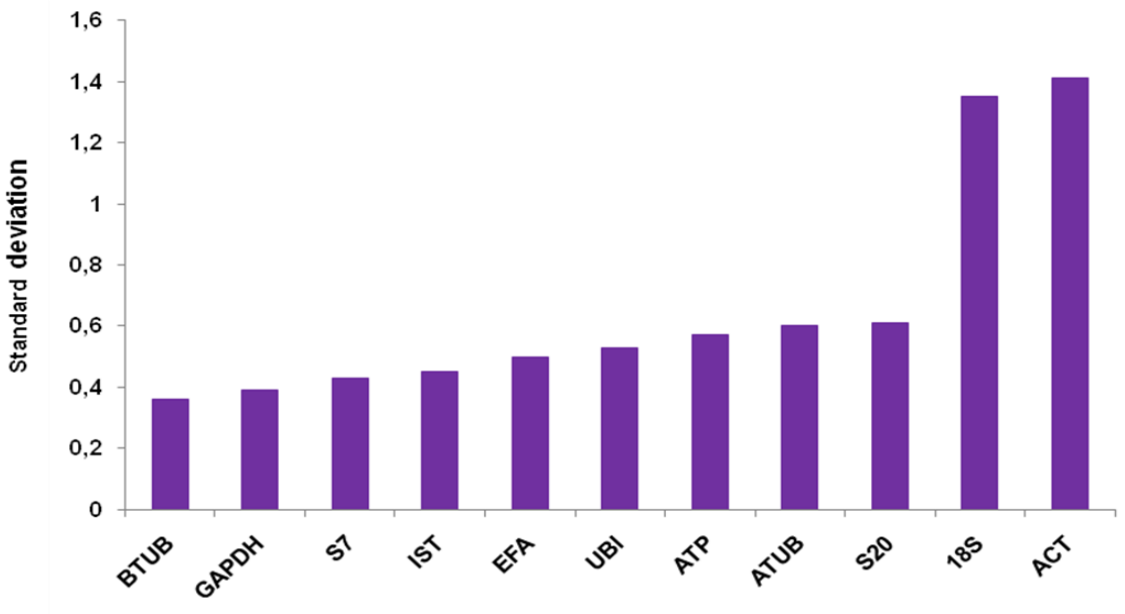

Figure 6.2. According to the mathematical approach of BestKeeper, the most stable RGs were those with the lowest standard deviation (SD) (y-axis). For gene acronyms see figure 6.1 caption.

GeNorm analysis (Vandesompele, et al., 2002) did not confirm the results of BestKeeper; the two most stable genes, with the lowest expression stability (M), were GAPDH and S7 (Figure 6.3). Pair-wise variation was subsequently calculated to evaluate the effect of adding another RG to those already analyzed. Below the cut-off value of 0.15 the inclusion of additional RGs was not required. According to our results (Figure 6.4), the two best RGs, GAPDH and S7, were sufficient for the analysis.

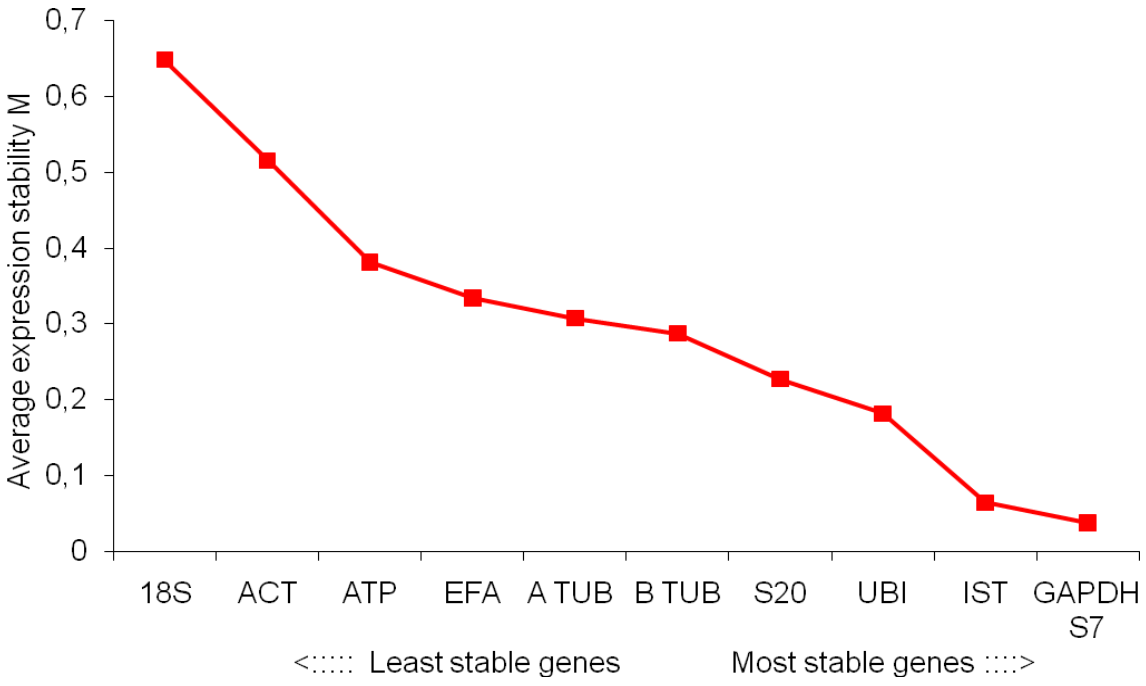

Figure 6.3. The stepwise exclusion of genes that are more variable among samples using the geNorm program. Data points represent the average expression stability values of remaining RGs. For gene acronyms see figure 6.1 caption.

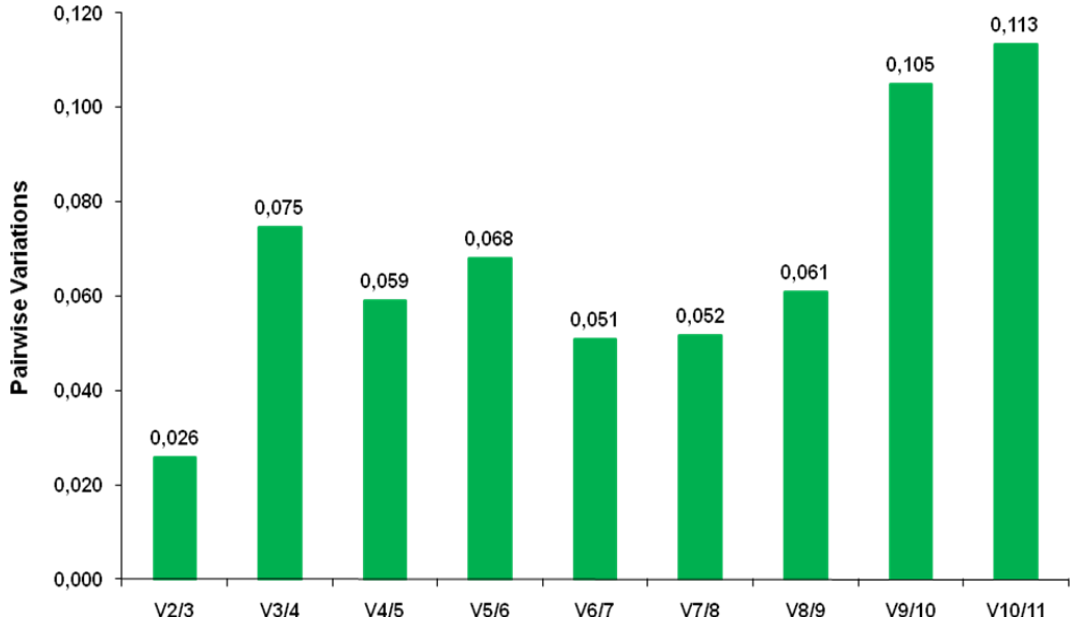

Figure 6.4. Pair-wise variation to evaluate the effect of adding another RG to those already analyzed. The inclusion of additional RGs was not required below the cut-off value of 0.15.

According to the statistical approach of NormFinder, our best candidate reference genes, with the lowest stability values, were S20, UBI and BTUB, as reported in figure 6.5. The rank pattern was different for the three softwares as summarized in table 6.1. We therefore decided to use as RGs those that were most stable for each one of the three software: S20, S7, GAPDH and BTUB. Moreover three RGs are suggested as the minimum significant number for reliable assessment of gene expression using qPCR (Bustin et al, 2010).

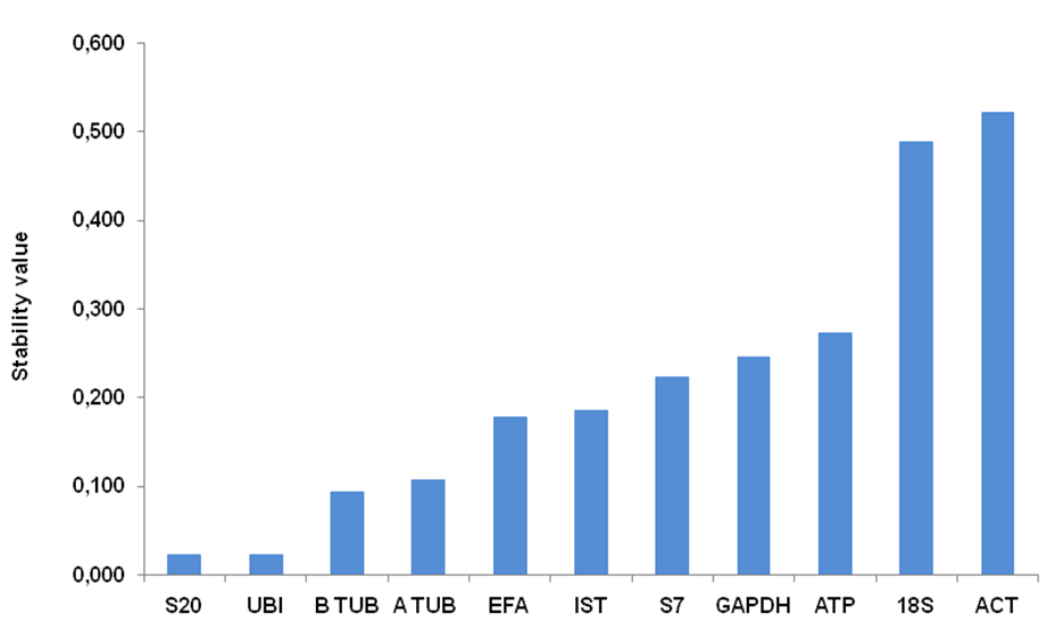

Figure 6.5. The NormFinder algorithm ranks the data set of candidate normalization genes according to their expression stability in a given experimental design. Blue bars represent the stability values of our candidate genes. For gene acronyms see figure 6.1 caption.

NORMFINDER	GENORM	BESTKEEPER
S20	**S7**	**B TUB**
UBI	**GAPDH**	GAPDH
B TUB	IST	S7
A TUB	UBI	IST
EFA	S20	EFA
IST	BTUB	UBI
S7	ATUB	ATP
GAPDH	EFA	A TUB
ATP	ATP	S20
18S	ACT	18S
ACT	18S	ACT

Table 6.1. The table ranks genes from the most stable to the least stable for each of the three softwares NormFinder, GeNorm and BestKeeper. For gene acronyms see figure 6.1 caption.

6.3.2 Expression level of genes of interest (GOI)

Expression levels of genes involved in generic stress response, phase I and phase II metabolic reactions, aldehyde detoxification and apoptosis regulation were analyzed in *Calanus helgolandicus* females fed on the toxin producing dinoflagellate *Karenia brevis* for three days. Females fed for three days on the control dinoflagellate *Prorocentrum minimum* were used as control for RT-qPCR analyses.

Expression levels of SOD, belonging to the primary defence system, were 2-fold up-regulated (students't-test, $p<0.05$, GraphPad Software) in copepods fed on *K. brevis*, while the other genes involved in generic stress response did not show significant changes (Figure 6.6).

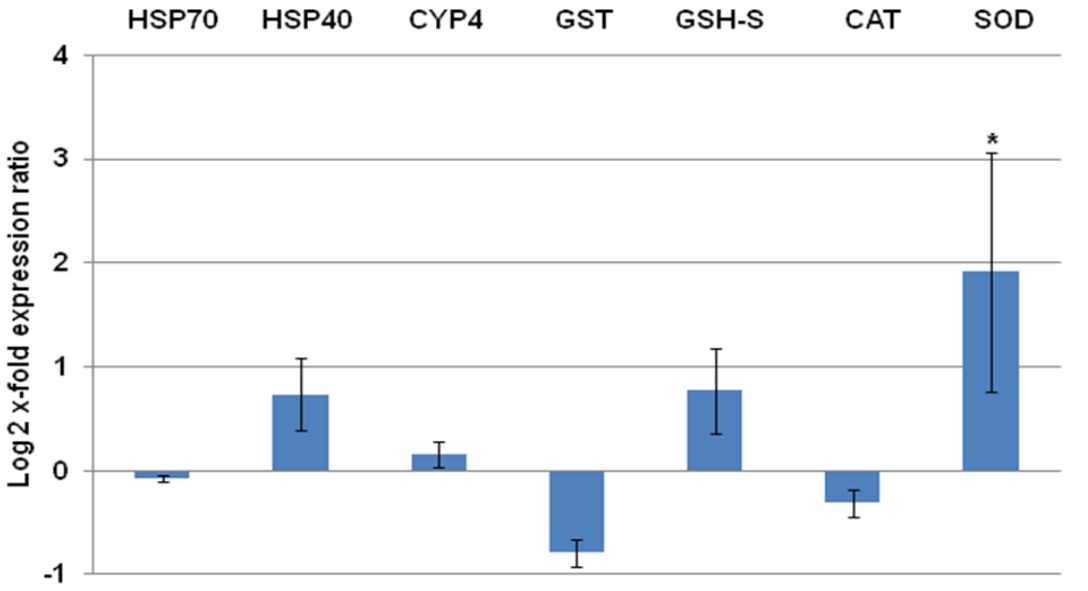

Figure 6.6. Expression levels (y-axis, Mean ± SD) of genes belonging to stress response and defense systems in *Calanus helgolandicus* females fed on the toxin producing dinoflagellate *Karenia brevis*. Females fed for three days on the control dinoflagellate *Prorocentrum minimum* were used as control. The best RGs, S20, S7, GAPDH and BTUB, were used to normalize RT-qPCR data.

Of the analyzed aldehyde dehydrogenases, ALDH6 was 2-fold up-regulated, even if with a high standard deviation denoting a high variability between the three replicates (SD=1.44) (students't-test, $p>0.05$, GraphPad Software) (Figure 6.7). The other ALDH expression levels did not show significant changes.

Of the genes involved in apoptotis regulation CAS expression levels increased, even if a high variability and standard deviation was observed (1.03) (students't-test, $p>0.05$, GraphPad Software). Interestingly, expression levels of actin significantly decreased (students't-test, $p<0.01$, GraphPad Software) (Figure 6.8).

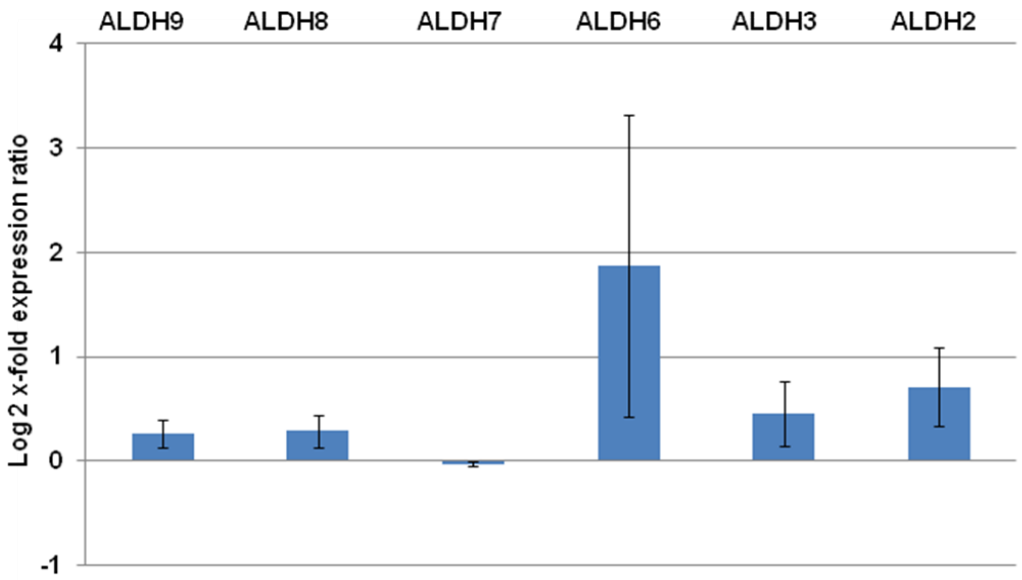

Figure 6.7. Expression levels (y-axis, Mean ± SD) of aldehyde dehydrogenases in *Calanus helgolandicus* females fed on the toxin producing dinoflagellate *Karenia brevis.* Females fed for three days on the control dinoflagellate *Prorocentrum minimum* were used as control. The best RGs, S20, S7, GAPDH and BTUB, were used to normalize RT-qPCR data.

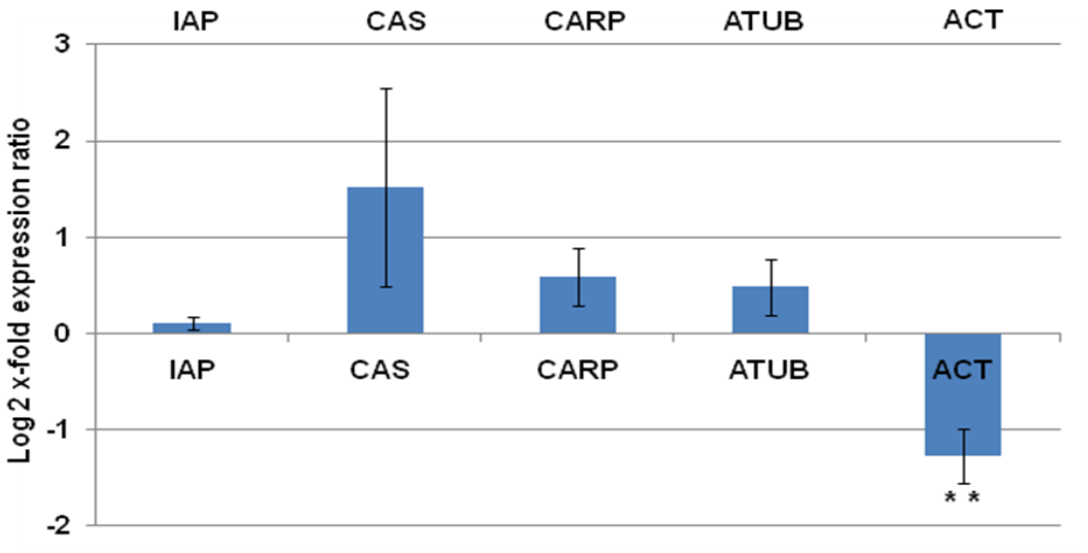

Figure 6.8. Expression levels (y-axis, Mean ± SD) of genes involved in apoptotis regulation and cytoskeleton structure in *Calanus helgolandicus* females fed on the toxin producing dinoflagellate *Karenia brevis*. Females fed for three days on the control dinoflagellate *Prorocentrum minimum* were used as control. The best RGs, S20, S7, GAPDH and BTUB, were used to normalize RT-qPCR data.

6.3.3 Microscopy

Image acquisition of eggs collected soon after spawning by females fed on the toxic dinoflagellate *K. brevis* for 3 days showed that cell divisions were impaired compared to eggs spawned by females fed for 3 days on the control dinoflagellate *P. minimum* (Figure 6.9). Apoptotic blebs were not visible and cytoplasm was released in the FSW denoting membrane cell breaks.

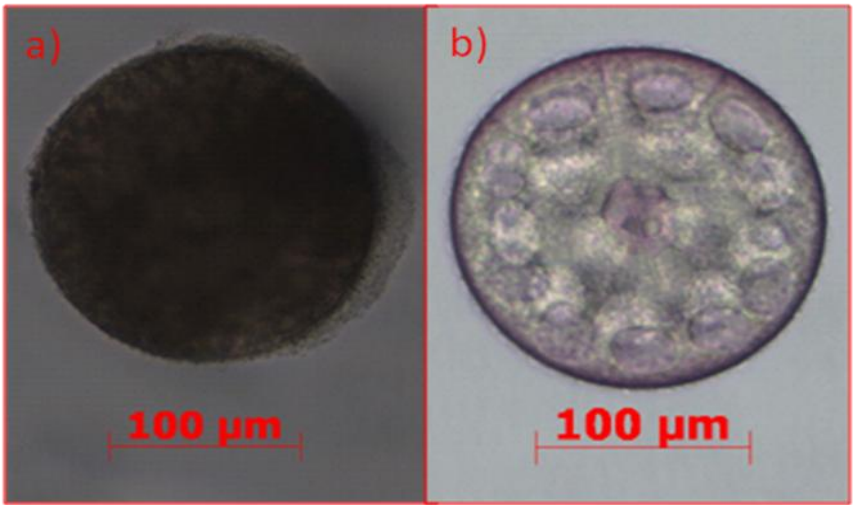

Figure 6.9. Confocal microscope images of eggs spawned by female fed on the dinoflagellate *Karenia brevis* for 3 days (a) or the dinoflagellate *Prorocentrum minimum* for 3 days (b).

6.4 Discussion

This chapter reports expression patterns of genes of interest in *Calanus helgolandicus* females fed on the brevetoxin-producing dinoflagellate *Karenia brevis.* Expression levels of the selected genes were different from the ones obtained for the same copepod species exposed to the oxylipin-producing diatom *Skeletonema marinoi*.

For the genes involved in generic stress response and primary defence systems, only SOD showed significant expression level changes in *C. helgolandicus* fed for 3 days on *K. brevis*. SOD was 2-fold up-regulated indicating an increase in the transformation of superoxide radicals ($*O^{2-}$) into hydrogen peroxide (H_2O_2). On the contrary, SOD was not affected in *C. helgolandicus* females during laboratory feeding experiments on the oxilipin-producing diatom *S. marinoi* (Lauritano, *et al.*, 2011b).

Of the ALDHs tested, ALDH6 was up-regulated in copepods fed for 3 days on *K. brevis*, even if there was high variability among triplicates (high standard deviation). The other ALDHs did not show significant changes. By contrast, ALDH6, ALDH8 and

ALDH9 were significantly down-regulated in copepods fed for 2 days on *S. marinoi*, while ALDH2, ALDH7 and ALDH9 expression levels increased after 120 h exposure to a diet of *S. marinoi*. Moreover, an increase in ALDH6, ALDH7 and ALDH8 levels was observed in the Atlantic *C. helgolandicus* population after 48 h of feeding on the same diatom species. It is widely known that ALDH are involved in protecting cells from the deleterious effects of xenobiotics and endogenous aldehydes such as those derived from lipid peroxidation (Canuto, *et al.*, 1994). Our results suggest that ALDH expression patterns depend on the tested algae, time of exposure and copepod population analyzed. However, these data indicate that oxidative responses were not activated in *C. helgolandicus* fed for 3 days on *K. brevis*, except for the anti-oxidant enzyme SOD.

Interestingly, ATUB and BTUB, that were very significantly affected in copepods fed for only 2-day on *S. marinoi* (Lauritano, *et al.*, 2011a), were very stable in animals fed on *K. brevis*. The BestKeeper software, in fact, assigned BTUB as best reference gene in *C. helgolandicus* fed for 3 days on *K. brevis*. Hence, in this case microtubule subunits, necessary for mitotic spindle formation to segregate chromosomes to dividing cells, were not impaired by a *K. brevis* diet.

In addition, ACT expression levels were reduced in copepod females fed on *K. brevis* for 3 days probably denoting a reduction in motile cell activity. Actin, in fact, generally interacts with the motor protein myosin and is involved in a wide range of cellular motile processes (Iwai, Uyeda, 2010). Whereas in copepods fed on *S. marinoi* tubulins could be possible target of the toxic diatom metabolites, our new data suggest that *K. brevis* toxins may have other cytoskeleton target, such as actin filaments.

Image acquisition of eggs collected soon after spawning by females fed on the toxic dinoflagellate *K. brevis* for 3 days showed that cell divisions were arrested. In addition, apoptotic blebs were not visible and cytoplasm was released in the

surrounding medium denoting membrane cell breaks suggesting that eggs die by necrosis and not by apoptosis. Apoptosis assays (e.g. TUNEL) and other approaches (e.g. protein analyses) may help clarify whether cell death is via apoptosis and/or necrosis and if the cell targets are in fact actin filaments. Moreover, time-series exposure of copepods to *K. brevis* will allow gene expression analyses after different times of exposure clarifying the expression patterns of selected genes of interest and their possible involvement in the copepod defense response to *K. brevis* toxins.

Chapter 7:

Conclusions

The molecular data reported in this thesis add considerably to the comprehension of copepod responses to toxic diets and in understanding the impact of these effects on local food webs. Expression level analyses using the sensitive reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) technique has allowed for the study of specific genes of interest (GOI) in various experimental conditions: copepods exposed to algae which produce or do not produce toxic metabolites in dinoflagellates (*Prorocentrum minimum*, *Rhodomonas baltica* or *Karenia brevis*) and diatoms (*Chaetoceros socialis* and *Skeletonema marinoi*) during field and laboratory experiments. In addition, the effects of the oxylipin producing diatom *Skeletonema marinoi* have been tested on two different *C. helgolandicus* populations: the Mediterranean population collected in the Adriatic Sea and the Atlantic population collected in the English Channel.

According to the results obtained, expression levels of specific GOI changed depending on the tested algae, time of exposure, copepod population analyzed and field/laboratory experiments. Moreover, the specific reponses may help to clarify if copepods are able to activate generic stress response genes when exposed to toxic diets or which are the copepod specific defense systems such as aldehyde, free fatty acid and free radical detoxification enzymes or apoptosis regulation genes activated or switched on/off in the presence of toxic algal metabolites.

The results provide molecular evidence for the toxic effects of certain diets on grazers. A synthesis of gene expression patterns in the copepod *C. helgolandicus* in the experimental conditions tested in this thesis is reported in table 7.1. After ingestion of the oxylipin-producing diatom *S. marinoi*, HSP70 and GST expression levels generally increase both in laboratory and field conditions, and in both Mediterranean and Atlantic populations, suggesting an increase in the generic stress response and antioxidant activity. CYP4 was generally down-regulated, except for the Mediterranean population during laboratory experiments, indicating a reduction in the free fatty acid metabolism. Of the ALDHs tested, ALDH3and ALDH8 expression levels decreased in both laboratory and field conditions, while ALDH7 levels increased, suggesting that ALDH7 was the isoform specific for diatom metabolite detoxification. ALDH6 was up-regulated only under laboratory conditions, but not in the field, during a natural bloom of the diatom *S. marinoi*, probably denoting an activity for metabolites produced in laboratory conditions or metabolites that in the field are very diluted. Interestingly, ALDH6 was up-regulated also in *C. helgolandicus* specimens exposed to the toxic dinoflagellate *K. brevis*. Finally, there was both down-regulation and up-regulation of BTUB, indicating condition-related responses. Other genes had no common responses between the experiments and showed gene expression patterns depending on the experimental condition tested (Table 7.1). The results show differences in the timing of activation of genes when *C. helgolandicus* is challenged with a toxic diet and highlights the different ability of Mediterranean and Atlantic populations to deal with toxic algae.

This exploratory study is currently being extended with the creation of a suppression subtractive hybridization (SSH) library for *Calanus helgolandicus* (Carotenuto et al., in preparation) which may further help to clarify which genes are differentially expressed in response to the ingestion of some diatom species. Given the

importance of diatom blooms in nutrient-rich aquatic environments these preliminary results offer a plausible explanation for the inefficient use of a potentially valuable food resource, the spring diatom bloom, by some zooplankton (Ianora et al., 2004, Miralto et al., 1999). Future studies on gene expression levels in *C. helgolandicus* specimens during natural diatom blooms in the Adriatic Sea, with more samplings during the same bloom, and with simultaneous measurements of phytoplankton composition and diatom chemical analyses may help identify the oxylipin concentrations in natural diatom blooms that trigger a response in copepod genes involved in detoxification systems or apoptosis regulation.

A multi-genic, integrated response to stressors may now be more accessible with -omics approaches, such as microarrays which have only recently been developed for copepod ecotoxicological studies. A 6K oligochip was used to study copper-induced detoxification in the copepod *T. japonicus* (Ki, *et al.*, 2009). Eichner *et al*. (Eichner, *et al.*, 2008) used a microarray approach in the sea lice *L. salmonis* during post molting maturation and egg production and a 12k microchip for *T. japonicus* is currently in progress for gene profiling after trace heavy metal and EDC exposure. Finally, high-throughout 454 pyrosequencing has been used to characterize a substantial fraction of the *T. californicus* transcriptome (Barreto, *et al.*, 2011) and a suppression subtractive hybridization library was performed for *C. finmarchicus* (Hansen, *et al.*, 2007). The advantage of -omics techniques is that they offer the possibility of simultaneously screening for a large number of genes and profiling the metabolic and defense patterns induced under different stress conditions. The disadvantage is that the activity of a protein does not always correlate to gene transcription, because of alternative splicing, regulator protein binding, and phosphorylation/dephosphorylation reactions that can activate/deactivate the protein (Nikinmaa, Waser, 2007). Nevertheless, mRNA

expression changes are an important early signal compared to other physiological parameters and/or phenotype changes. The existing ecotoxicological data on copepods highlight the diversified mechanisms and high number of genes that may be involved in the stress response of this group of aquatic crustaceans. The identification of new genes, for example using cDNA microarrays or new generation sequencing, and the application of new tools, such as functional proteomic approaches, may allow for a more comprehensive overview of how copepods respond to specific stressors in the laboratory, but also to predict the response under natural environmental conditions and the effects of these responses on higher trophic levels.

	MEDITERRANEAN SEA				ATLANTIC OCEAN (ROSCOFF)				BLOOM 2010		BLOOM 2011			*K. brevis*
	12 H	24 H	48 H	120 H	12 H	24 H	48 H	120 H	APRIL	MAY	15_3	29_3	9_4	
HSP70	DOWN	UP			DOWN	UP			UP	UP		UP	UP	
HSP40			DOWN	UP										
CYP4							DOWN		DOWN		DOWN	DOWN	DOWN	
GST	UP				UP		DOWN	UP	UP	UP		UP	DOWN	
GSH-S			DOWN	UP							UP			
SOD									DOWN			UP	UP	UP
CAT					UP	UP			DOWN					
ALDH9			DOWN	UP										
ALDH8			DOWN			UP		DOWN	DOWN					
ALDH7				UP		UP				UP		UP	UP	
ALDH6	UP		DOWN			UP		UP						UP
ALDH3	DOWN			UP	DOWN				DOWN					
ALDH2				UP					DOWN					
CARP				UP										
CAS			DOWN	UP										UP
IAP			DOWN					DOWN	DOWN					
A TUB	UP		DOWN	UP										
B TUB	UP		DOWN	UP	UP	UP				UP		DOWN	DOWN	
ACT														DOWN

Table 7.1. Synopsis of the expression patterns of the genes of interest in the experimental conditions reported in this thesis. For gene abbreviations see Appendix 4. HSP70, GST, ALDH6 and ALDH7 (in red) were often up-regulated, while CYP4, ALDH8 and ALDH3 (in blue) were often down-regulated both in the field and lab experiments.

APPENDIX 1: RNA and DNA extraction protocols

RNA EXTRACTION

1. Homogenization Tissues: Homogenize animals in 0.500 ml of TRIZOL® Reagent (Invitrogen) per 1/40 individuals using the Qiagen Tissue Lyser and Tungsten Carbide Beads (3mm) (Quigen)
2. Centrifugation at 12,000 × g for 10 minutes at 4°C
3. Add 0.1 ml of chloroform per 0.500 ml of TRIZOL
4. Shake tubes vigorously by hand for 15 seconds and incubate them at 25°C for 5 minutes
5. Centrifugation at 12,000 × g for 15 minutes at 4°C (Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase)
6. Transfer the aqueous phase to a fresh tube, and save the organic phase if isolation of DNA is desired.
7. Add a quantity of chloroform identical to the total volume obtained at this step.
8. Centrifugation at 12,000 × g for 10 minutes at 4°C
9. Transfer the aqueous phase to a fresh tube
10. Repeat step 7, 8 and 9 two times
11. Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use a quantity of isopropyl alcohol identical to the total volume obtained at this step
12. Incubate samples at 25°C for 10 minutes

13. Centrifuge at 12,000 × g for 10 minutes at 4°C
14. Wash RNA pellet two times with 0.500 ml 75% ethanol
15. At the end of the procedure, briefly air-dry the RNA pellet and dissolve RNA in 10 µl RNase-free

DNA EXTRACTION

1. Remove the remaining aqueous phase overlying the interphase (RNA extraction protocol step 6)
2. Precipitate the DNA from the interphase and organic phase with ethanol. Add 0.150 ml of 100% ethanol per 0.500 ml of TRIZOL used for the initial homogenization, and mix samples by inversion.
3. Next, store the samples at 25°C for 2-3 minutes
4. Centrifugation at no more than 2,000 × g for 5 minutes at 4°C
5. Remove the phenol-ethanol supernatant
6. Wash the DNA pellet twice in a solution containing 0.1 M sodium citrate in 10% ethanol
7. (Use 0.500 ml of the solution per 0.500 ml of TRIZOL used for the initial homogenization)
8. At each wash, store the DNA pellet in the washing solution for 30 minutes at 25°C

   (with periodic mixing)
9. Centrifuge at 2,000 × g for 5 minutes at 4°C

10. Suspend the DNA pellet in 75% ethanol (1 ml of 75% ethanol per 0.500 ml TRIZOL)
11. Store for 10-20 minutes at 25°C (with periodic mixing)
12. Centrifuge at 2,000 × g for 5 minutes at 4°C
13. Air dry the DNA 5 to 15 minutes in an open tube
14. Dissolve DNA in 8 mM NaOH
15. Centrifuge at 12,000 × g for 10 minutes at 4°C
16. Transfer the supernatant containing the DNA to a new tube

APPENDIX 2: Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) details

The fluorescent dye SYBR GREEN was used to value expression levels of the selected genes by RT-qPCR. This dye shows maximal fluorescence only with double-strand DNA and was used to detect our amplicons. Fluorescence was monitored once per cycle after product extension and increased above background fluorescence at a cycle number that depended on the initial template concentration.

For Chapter 2 and 3:

RT-qPCR was performed in Bio-Rad 96-well reaction full skirted white plates with Microseal 'B' Adhesive Seals (BioRad) in a *Chromo4 TM Real-time Detector* (Biorad) thermal cycler, whereas fluorescence was measured using Opticon Monitor 3.1 (Biorad). The PCR volume for each sample was 25 µl, with 1X of *Fast Start SYBR Green Master Mix* (Roche), 2 µl of cDNA template and 0.7 pmol/µl 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 sec and 60°C for 1 min, 72°C for 5 min. The programme was set to reveal the melting curve of each amplicon from 60°C to 95°C, and read every 0.5°C.

For Chapter 4, 5 and 6:

RT-qPCR was performed in MicroAmp Optical 384-Well reaction plate (Applied Biosystem) with Optical Adhesive Covers (Applied Biosystem) in a Viia7 Real Time PCR System (Applied Biosystem). The PCR volume for each sample was 10 µl, with 5µl of *Fast Start SYBR Green Master Mix* (Roche), 1 µl of cDNA template and 0.7 pmol/µl 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 sec and 60°C for 1 min, 72°C for 5

min. The programme was set to reveal the melting curve of each amplicon from 60°C to 95°C, and read every 0.5°C.

For all the Chapters:

In RT-qPCR assays, the optimal quantity of template of 1:100 was assessed using serial dilutions, ranging from 1:1 to 1:10000.

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. Five serial dilutions of cDNA were used to determine reaction efficiencies for all primer pairs. These efficiencies (Table 2.2) were calculated generating for each oligonucleotide pair standard curves with at least five dilution points by using the Cycle Threshold (Ct) value versus the logarithm of each dilution factor and using the equation $E=10^{-1/slope}$. Primer efficiencies (E) ranged from 89% to 100% while the linear correlation coefficient (R^2) ranged from 0.9704 to 0.9998 (Table 2.2). Each primer pair generated different and individual fluorescence patterns in the qPCR kinetics (Melting Curve). A 1:100 template dilution (4 ± 2 ng) was used which allowed almost all gene amplifications to fit in the optimal read window (from 15 to 25 cycles). Only a single peak in the melting-curve analyses of all genes was identified, confirming a gene-specific amplification and the absence of primer-dimers.

Three different algorithms were utilized to identify the best reference genes in our experimental design: BestKeeper (Pfaffl, *et al.*, 2004), geNorm (Vandesompele, *et al.*, 2002) and NormFinder (Andersen, *et al.*, 2004) in chapter 2 and 6.

To study the expression of each target gene relative to the most stable RGs, we used REST tool (Relative expression software tool) (Pfaffl, *et al.*, 2002). This tool used a mathematical model based on the PCR efficiencies and the mean crossing point deviation between the sample and the control group. The advantage of REST is that this

software tool tests the group differences for significance with the Pair-Wise Fixed Reallocation Randomization Test (Pfaffl, *et al.*, 2002).

APPENDIX 3: Solutions

TBE 5x (1L)

Tris 54 g

Acido borico 27.5 g

EDTA 0.5 M (pH 8.0) 20 ml

TBE 1x (1L)

TBE 5x 100 ml

Distilled water

APPENDIX 4: Abbreviations

- A TUB: Alpha tubulin (α tub)
- ABC: adenosine triphosphate-binding cassette
- ACT: β-actin
- ALDH: aldehyde dehydrogenase
- ATP: adenosine triphosphate
- ATPs: adenosine-3-phosphate synthase
- B TUB: Beta tubulin (β tub)
- BLAST: Basic local alignment search tool
- BPA: bisphenol A
- BSA: Bovine sieric albumine
- *C. helgolandicus: Calanus helgolandicus*
- *C. finmarchicus: Calanus finmarchicus*
- *C. socialis*: *Chaetoceros socialis*
- CAS: cellular apoptosis susceptibility protein
- CARP: cell cycle and apoptosis regulatory 1 protein
- CAT: catalase
- cDNA: complementary DNA
- COI: Cytochrome Oxidase I
- Ct: Cycle Threshold
- Cu: copper
- CYP450: cytochrome P450
- CYP4: cytochrome P450 family 4 monooxygenases
- d: day/s

- dNTP: deossinucleotides
- DEA: diethanolamine
- DSP: Diarrhetic Shellfish Poisoning toxins
- E: primer efficiencies
- EDC: endocrine disruptor chemicals
- EFA: elongation factor 1α
- EMB: emamectin benzoate
- EST: expressed sequence tag
- FSW: filtered sea water
- GAPDH: glyceraldehyde-3-phosphate dehydrogenase
- GOI: gene of interest
- GSH-S: glutathione synthase
- GST: glutathione S-transferase
- h: hour/s
- HIST: histone 3
- HABs: harmful algal blooms
- H_2O_2: hydrogen peroxide
- HSPs: heat shock proteins
- IAP: Inhibitor of apoptosis family
- *K. brevis: Karenia brevis*
- LPO: lipid peroxidation
- MEA: mono ethanol amine
- min: minute/s

- MIQE: Minimum Information for Publication of Quantitative Real-Time PCR Experiments
- MTs: microtubules
- MXR: multixenobiotic resistance system
- NP: 4-nonylphenol
- NSP: neurotoxic shellfish poisoning
- NTC: no-template negative controls
- OP: 4-t-octylpheno
- Pb: lead
- PbTx-2: Brevetoxin
- *P. minimum*: *Prorocentrum minimum*
- PRO: *Prorocentrum minimum*
- PSP: Paralytic Shellfish Poisoning toxins
- PUAs: polyunsaturated aldehydes
- PUFAs: polyunsaturated fatty acids
- R^2: correlation coefficient
- *R. baltica*: *Rhodomonas baltica*
- REST: Relative expression software tool
- RG: reference gene
- RHO: *Rhodomonas baltica*
- ROS: reactive oxygen species
- RT-qPCR : Reverse transcripatase-quantitative polymerase chain reaction
- S7: ribosomal protein S7
- S20: ribosomal protein S20

- SD: Standard deviation
- SKE: *Skeletonema marinoi*
- *S. marinoi*: *Skeletonema marinoi*
- SOD: dismutase
- ST: station
- *T. japonicas*: *Tigriopus japonicus*
- T_m: melting temperature
- TUNEL: Terminal Transferase dUTP Nick End Labeling Assay
- WSFs: water-soluble fractions of oil

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