

Age-dependent expression of stress and antimicrobial genes in the hemocytes and siphon tissue of the Antarctic bivalve, *Laternula elliptica*, exposed to injury and starvation

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Abstract Increasing temperatures and glacier melting at the Western Antarctic Peninsula (WAP) are causing rapid changes in shallow coastal and shelf systems. Climate change-related rising water temperatures, enhanced ice scouring, as well as coastal sediment runoff, in combination with changing feeding conditions and microbial community composition, will affect all elements of the nearshore benthic ecosystem, a major component of which is the Antarctic soft-shelled clam *Laternula elliptica*. A 454-based RNA sequencing was carried out on tissues and hemocytes of *L. elliptica*, resulting in 42,525 contigs, of which 48 % was assigned putative functions. Changes in the expression of putative stress response genes were then investigated in hemocytes and siphon tissue of young and old animals subjected to starvation and injury experiments in order to investigate their response to sedimentation (food dilution and starvation) and iceberg scouring

(injury). Analysis of antioxidant defense (*Le*-SOD and *Le*-catalase), wound repair (*Le*-TIMP and *Le*-chitinase), and stress and immune response (*Le*-HSP70, *Le*-actin, and *Le*-theromacin) genes revealed that most transcripts were more clearly affected by injury rather than starvation. The upregulation of these genes was particularly high in the hemocytes of young, fed individuals after acute injury. Only minor changes in expression were detected in young animals under the selected starvation conditions and in older individuals. The stress response of *L. elliptica* thus depends on the nature of the environmental cue and on age. This has consequences for future population predictions as the environmental changes at the WAP will differentially impact *L. elliptica* age classes and is bound to alter population structure.

Keywords Western Antarctic Peninsula · Transcriptome · Climate change · Antioxidants · Immune response

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Introduction

The molecular immune repertoire and stress response of marine water and freshwater bivalves is still poorly understood (summarized recently by Tomanek 2011, Venier et al. 2011, and Philipp et al. 2012b). Bivalves rely exclusively on the innate immune system (Bachere et al. 1995; Canesi et al. 2002). Hence, when the physical barriers of shell and epithelia have been breached as a result of injury and/or infection, invading microorganisms, nonself particles, and cell debris are recognized and eliminated by humoral and cellular mechanisms. Humoral responses comprise the permanent or inducible biosynthesis of antimicrobial peptides or other defensive peptides (Bettencourt et al. 2007). Cellular reactions include phagocytosis and the generation of reactive oxygen species (ROS)

(“oxidative burst”). These are mediated by the hemocytes (Donaghy et al. 2009; Husmann et al. 2011; Pruzzo et al. 2005). These cells have key functions not only in immunity but also in the maintenance of physiological homeostasis. They are involved in the inflammatory response, wound repair, shell formation, transport of nutrients, digestion, as well as excretion of biological active molecules such as enzymes or antimicrobial peptides (Canesi et al. 2002; Donaghy et al. 2009; Kadar 2008). In order to regenerate integrity and function in organs after injury, hemocytes migrate towards the wound and infiltrate the affected tissue (de Eguileor et al. 1999; Ottaviani et al. 2010). A granulated tissue is produced, which seals the wound with the synthesis of extracellular matrix components and, finally, reepithelialization can occur (Ottaviani et al. 2010). The ability to mount such cellular defense reactions in order to maintain organism homeostasis under environmental disturbance is crucial for survival (Hawley and Altizer 2011; Mydlarz et al. 2006). However, it is important to note that the immunocompetence of bivalves is related to general ecological factors such as temperature, salinity, and food availability (Fisher et al. 1987; Hawley and Altizer 2011; Butt et al. 2007; Hegaret et al. 2004). It is also affected by artificially induced stressors such as injury and mechanical disturbance (Husmann et al. 2011; Ballarin et al. 2003) or xenobiotics and pollution (Oliver et al. 2001; Chu et al. 2002). Indeed, the latter was found to promote viral, bacterial, and protozoan diseases, potentially causing extensive mass mortalities in bivalves (Morley 2010; Beaz-Hidalgo et al. 2010; Hawley and Altizer 2011).

The effects of environmental stress on the cellular immunity of bivalves have been largely investigated in terms of hemocyte abundance or phagocytotic and enzymatic activity. For example, differential food quality was found to influence phagocytotic activities, ROS production, and abundance of free-floating hemocytes in *Crassostrea gigas* and *Ruditapes philippinarum* (Delaporte et al. 2003, 2006). In large-sized *Laternula elliptica*, reduced hemocyte numbers were detected in animals subjected to starvation treatments compared to constant feeding (Husmann et al. 2011). Further, in the clam *R. philippinarum* (Oubella et al. 1993) and the Sydney rock oyster *Saccostrea glomerata*, starvation reduced hemocyte numbers and decreased phenol oxidase, peroxidase, and acid phosphatase activities (Butt et al. 2007). Similarly, mechanical disturbance (shaking) reduced hemocyte abundance and oxidative burst activity in abalones *Haliotis tuberculata* and blue mussels *Mytilus edulis* (Bussell et al. 2008; Malham et al. 2003). In contrast, increased hemocyte numbers were observed in response to shell injury in deep-sea vent mussels *Bathymodiolus azoricus* (Kadar 2008) and in the Antarctic clam *L. elliptica* (Husmann et al. 2011). This difference in response may be due to the fact that shell injury causes a major breach in animal integrity, while starvation may produce energetic trade-offs in animal biochemistry.

A recently investigated aspect of the bivalve defense response is the regulation of molecular effectors such as functional peptides and proteins in hemocytes and soft body tissues (Koutsogiannaki and Kaloyianni 2010; Tomanek 2011). Other proteomic/transcriptomic studies in bivalves point towards a common set of stress-induced proteins (Tomanek 2011). These comprise heat shock proteins (HSPs) involved in the stabilization of proteins (Clark et al. 2008a; Santoro 2000), as well as molecules participating in oxidative stress regulation (Canesi et al. 2010; Monari et al. 2008; Park et al. 2009), tissue damage repair (De Decker and Saulnier 2011; Montagnani et al. 2001), tissue development (Badariotti et al. 2006, 2007b; Tirape et al. 2007), or antimicrobial defense (Xu et al. 2010).

At the Western Antarctic Peninsula (WAP) region, recent rapid aerial warming has caused profound environmental changes, including warming of the water in shallow coastal and shelf areas and rapid glacier disintegration (Turner et al. 2009; Cook et al. 2005; Schloss et al. 2012). Glacier melt water streams carry high amounts of terrestrial mineral suspensions into the marine coastal environment, and therefore, higher levels of glacier melt result in increased near-shore marine sedimentation loads (Dominguez and Eraso 2007; Schloss et al. 2012). The calving of glacial fronts and ice shelves produces increased amounts of floating brash ice and icebergs and hence more ice scouring in shallow coastal areas (Turner et al. 2009; Barnes and Souster 2011; Brown et al. 2004). Such changes will have marked consequences for benthic animals colonizing coastal areas around the WAP (Barnes and Conlan 2007; Barnes and Kaiser 2007).

To investigate the implications of these changes on, and also predict future responses of the nearshore marine benthic ecosystem, this study investigated the effect of high sediment concentration and mechanical injury on a major component of the Antarctic benthic ecosystem, the filter-feeding bivalve *L. elliptica*. This long-lived (36 years life span) clam is endemic to the maritime Antarctic and one of the most abundant sentinel species of the infauna in soft bottom habitats (Philipp et al. 2005a, 2011). Due to its circumpolar distribution and contribution to benthopelagic coupling, it plays a key role within the Antarctic coastal ecosystem (Ahn 1997; Momo et al. 2002; Philipp et al. 2011).

The aim of these experiments was to address the potential effects of altered food supply (by enhanced sedimentation and dilution of planktonic food with mineral suspension), and tissue injury by scouring ice blocks on a keystone species.

Previous data on this species revealed a higher susceptibility to environmental stress in older age classes of *L. elliptica*. Older *L. elliptica* exhibited reduced metabolic rates during exposure to high sediment loads and featured lower survival rates after injury compared to younger

cohorts (Philipp et al. 2011). Furthermore, they are reported to be more sensitive to increased water temperatures (Peck et al. 2007) and have a more limited ability to reburrow into the sediment when unearthed by icebergs (Morley et al. 2007; Peck et al. 2004; Philipp et al. 2011). They also exhibit lower oxidative defense capacities and higher levels of oxidative damage (Philipp et al. 2005a), and their hemocytes are less able to mount an oxidative burst response compared to hemocytes of younger specimens (Husmann et al. 2011). If this differing physiological fitness and increased sensitivity to environmental challenge found in older animals lead to selective mortality in the corresponding age classes, it is expected that the age and size structure of *L. elliptica* populations will change in the near future.

This study investigated the changes in gene expression levels in response to injury and starvation in younger and older individuals of *L. elliptica* to identify age-specific responses. A 454-based high-throughput sequencing (GS FLX, Roche–454 Life Sciences) was used to generate an extensive RNA sequence database to enable a more comprehensive choice of candidate genes for in-depth investigations. The expression changes of selected candidate genes involved in the general stress and immune response were studied in the hemocytes and siphon tissue of young and old *L. elliptica* individuals from starvation and injury experiments. The potential impact of these factors on future population structures is discussed.

Material and methods

Sampling of animals

L. elliptica individuals were collected by divers in Potter Cove, King George Island, Antarctic Peninsula (62°14' S, 58°40' W), between November 2008 and February 2009 at 7–15 m depth. Animals were classified as “large” with 7.2 ± 0.5 cm (mean \pm SD) shell length and “small” with 4.8 ± 0.5 cm shell length. Approximate ages of the animals were estimated from shell size using a *L. elliptica* von Bertalanffy growth model from Potter Cove (Philipp et al. 2008). Ages within the group of “small” individuals ranged between 4 and 6 years, and in the “large” group, >11 years. At the Dallmann Laboratory on the Argentinean Station Carlini (formerly named Jubany), animals were kept in four 150-L tanks (40 small and 40 large animals per tank) for 10 days in aerated, natural seawater from Potter Cove at a constant temperature of 1 °C without sediment. Every second day, 50 % of the water in the holding system was renewed to ensure good water quality. Following this acclimation period, individuals from both size/age groups were exposed to different stress treatments.

Stress experiments

Small and large *L. elliptica* were subjected to starvation and injury. Half of the animals from each size group were starved by keeping them in filtered seawater of 0.5 μ m final pore size (treatment “starvation”). The other half of the group was kept as “fed controls” in unfiltered seawater which was additionally enriched with dissolved and particulate nutrients (treatment “food”). The mixed diet consisted of live microalgae, as well as “artificial detritus,” i.e., freeze-dried and ground macroalgae (*Ascoseira mirabilis*) from Potter Cove, as well as krill and red bloodworms obtained from Tetra (TetraDelica; Tetra, Melle, Germany). Microalgae were collected with fine nets in Potter Cove and cultured in 2-L bottles at 1 °C. Animals were fed every second day (1 g macroalgae and zooplankton per tank, microalgae). Concentrations of added detritus were close to summer maxima under natural conditions according to Atencio et al. (2008).

After 21 days, 50 % of the small and large animals from the starvation and food treatment were artificially injured. Both valves of the animals were cracked by a soft blow with a blunt tool (wrench) in such a way that also the mantle was injured. Additionally, the siphon was cut at two places. After injury infliction, the animals were allowed to recover in their tank of origin under the respective food condition. Two days after the injury event, i.e., at day 23 after starting the food treatments, hemocyte cells and tissues were sampled for gene expression analysis from all groups. Additional samples were taken at day 44 after the initial start of the different food treatments, i.e., in case of the injured animals after 21 days of regeneration from the injury. Hemolymph fluid containing the hemocyte cells was individually collected by inserting a 16-gauge needle with a 10-ml syringe into the posterior adductor muscle and slowly withdrawing the hemolymphatic fluid. Two aliquots of 1.5 ml each were sampled and immediately centrifuged 10 min at 1,000 \times g and 2 °C. The supernatant was discarded and the hemocyte pellet flash-frozen in liquid nitrogen. Subsequently, animals were sacrificed and the different tissues flash-frozen in cryovials in liquid nitrogen. The hepatosomatic index (HSI) was calculated from digestive gland fresh weight and total tissue fresh weight to provide indications on the physiological state of the experimental animals as:

$$\text{HSI} = (\text{fresh weight of digestive gland} / \text{total tissue fresh weight}) \times 100$$

Frozen samples were transported to the Institute of Clinical Molecular Biology (ICMB) in Kiel, where they were kept at –80 °C until further processing.

L. elliptica RNA sequence database generation

In order to maximize the number of expressed transcripts and facilitate the identification of candidate genes involved in injury, immune system, and general stress response processes, samples from both *L. elliptica* age groups and the different treatments were used for RNA sequence database generation. Total RNA was extracted separately from samples of the digestive gland, gill, and siphon (one small and one large animal each from the treatment “starvation/acute injury” and “food/acute injury”), as well as from hemocyte cells (four small animals from treatment “starvation,” five small from “food,” four large from “starvation/acute injury,” and four small as well as four large from “food/acute injury”). Hemocytes were selected because they are major components of the immune system and siphon as it was directly inflicted with injury in the experiment. Digestive glands were chosen because they contain major components of the digestive system and metabolism of the animals. Gills were included as they constitute a major site of contact to ingested particulate matter, microbiota, or dissolved nutrients. Tissues were ground in liquid nitrogen, and the total RNA of hemocyte cells and tissues was extracted using the Qiagen RNeasy kit in combination with Qiagen QiaShredder columns (Qiagen, Hilden, Germany), including DNase digestion. According to the manufacturer’s instructions, the quality of the extracted RNA was checked photometrically by a NanoDrop spectrophotometer (PepLab, Erlangen, Germany), and only samples showing 260/280 nm absorbance ratios of >2.2 were further processed. Equal amounts of total RNA were combined into to a single pool, and mRNA was isolated using the Oligotex mRNA purification kit (Qiagen, Hilden, Germany). Two cDNA samples were generated from the extracted mRNA by using two alternative ways: (1) by the BD SMART PCR cDNA Synthesis Kit (Clontech Laboratories, Palo Alto, CA, USA) and (2) by the GS FLX Titanium Series cDNA Rapid Library Preparation Method Protocol (October 2009, Rev. January 2010, Roche).

Pyrosequencing, assembly, and processing of 454 sequences

cDNA was sequenced on the Genome Sequencer FLX System (454 Life Sciences, Branford, CT, USA) using titanium chemistry according to the manufacturers’ protocol at the ICMB. The resulting 454 sequences were extracted from FLX output files using the “sffinfo” script from Roche. Prior to assembly, polyA tails, SMART primer sequences, 454 adapter sequences, and sequences of bad quality (quality score < 11) were removed from the data set using “seqclean” and “cfn2qual” software (The Gene Index [TGI] Project). Read sequences < 40 bp after filtering were also discarded. Of the initial 500,954 sequences, 489,924 remained and were assembled using the “GS De Novo Assembler 2.3” software (NEWBLER, Roche–454 Life Sciences). Assembly

parameters were minimum overlap length=40 and minimum overlap identity=90. The resulting contigs and singletons were further repeatedly assembled using the TGICL (Cap3) software with the following filter parameters: minimum overlap length, 40–360 bp; minimum overlap identity, 90–100 %. For a last contig refinement, the initial filtered reads were mapped against the assembled contigs using the AMOScmp software (<http://sourceforge.net/apps/mediawiki/amos/index.php?title=AMOScmp>).

An additional data set of 1,034,154 sequences, generated from *L. elliptica* mantle tissue using the 454 technology, was provided by the British Antarctic Survey (BAS); for details, see Clark et al. (2010). After cleaning as described previously, 808,460 sequences remained. The combined 1,298,384 reads of the first (ICMB) and second (BAS) data sets were mapped against the previously generated contigs using AMOScmp. The resulting contigs and singletons were then repeatedly assembled using the TGICL (Cap3) software as described previously. Finally, all reads were mapped again against the assembled contigs using the AMOScmp software. This resulted in the final contig data set.

RNA sequence database annotation and identification of candidate genes

Putative gene names were assigned to the assembled contigs by BLASTx analysis against the UniProtKB/Swiss-Prot protein database (UniProt Knowledgebase, <http://www.expasy.org/sprot>) and tBLASTx against the NCBI nonredundant protein (nr) database (<http://www.ncbi.nlm.nih.gov>) with $e \leq 10^{-3}$ as cutoff value. Conserved protein domains were identified using InterProScan (Hunter et al. 2009).

Annotation data were loaded together with contig sequence information into T-ACE, a software tool for the processing of large RNA sequence data sets of nonmodel organisms (Philipp et al. 2012a). Candidate genes for metabolic, injury, immune, and general stress response in *L. elliptica* were then identified by key word searches and sequence similarity searches in the annotated database. Candidate contigs were manually verified against the NCBI nr/nt and UniProtKB/Swiss-Prot database using tBLASTx and BLASTx, respectively. The protein domain structure of the different contigs was further investigated using the NCBI conserved domain architecture retrieval tool cDART (<http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi>) and compared to the conserved domain structure for the orthologous gene as defined by HomoloGene (<http://www.ncbi.nlm.nih.gov/homologene>).

Gene expression analysis

Expression changes of selected candidate genes were investigated in hemocytes and siphon tissue of starved and/or

injured *L. elliptica* individuals by quantitative real-time polymerase chain reaction (qRT-PCR) on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green (Power SYBR Green PCR Master Mix, Applied Biosystems, Foster City, CA, USA). Tissues were ground in liquid nitrogen, and total RNA was extracted via the standard TRI reagent protocol (Sigma-Aldrich, St. Louis, MO, USA) in combination with Qiagen QiaShredder columns (Qiagen, Hilden, Germany), followed by cleanup using the Qiagen RNeasy columns; all according to manufacturers' instructions including DNase digestion. The quality and quantity of the extracted mRNA was checked as described previously, and only samples showing 260/280 nm absorbance ratios of >2.1 were used for cDNA generation with the Advantage RT-for-PCR Kit (Clontech, Heidelberg, Germany). Primers targeting candidate genes were designed using Primer3 (Rozen and Skaletsky 2000) and checked for hairpins, self-dimers, and heterodimers with OligoAnalyzer 3.1 (Integrated DNA Technologies, Coralville, IA, USA) (Table 1). To ensure primer specificity and verify contig assembly, semiquantitative PCR was performed with Advantage Taq2 Polymerase (Clontech, Heidelberg, Germany) and the appropriate PCR conditions. The presence and size of the PCR products were confirmed via agarose gel electrophoresis. The cDNA was isolated from the gel with the Wizard SV Gel and PCR Clean-Up System (Promega, Fitchburg, WI, USA) and Sanger sequenced using Big Dye Terminator chemistry (Applied Biosystems, Foster City, CA, USA). The resulting Sanger sequences were compared with the respective contig from the RNA sequence database using Sequencher version 4.5 (GeneCodes, Ann Arbor, MI, USA). qRT-PCR analysis for each sample was performed in triplicate. The conditions were as follows: 50 °C (2 min), 95 °C (10 min), 45 cycles of 95 °C (15 s) and 60 °C (1 min). To check for artifacts, a melting curve was generated for each primer pair. The comparative CT method (delta Ct) for the relative quantification of gene expression was used (Livak and Schmittgen 2001) and data were normalized using 18S ribosomal RNA (18S rRNA) as the reference gene.

Statistical analysis

Gene expression data sets and data of the HSI were tested for Gaussian distribution using the Kolmogorov–Smirnov test. Homogeneity of variances was assessed by the Brown–Forsythe test for equal variances. When necessary, data were transformed (square root, log, LN, or $-1 \times \log Y$) to achieve normal distribution and homogeneity of variances.

In case of normally distributed data, statistical analysis was performed using the software package STATISTICA for Windows version 7.1 (StatSoft Inc., Tulsa, OK, USA). Effects of time and treatment were evaluated by two-way analysis of variance (ANOVA) (variables “time” and

“treatment”) and the Bonferroni post hoc test. In case of non-normally distributed data, these were analyzed using the nonparametric “adonis” Permutational Multivariate Analysis of Variance using distance matrices (Anderson 2001) of the R software package “vegan” (Dixon 2003; Oksanen et al. 2011), R statistic software version 2.13.1.

Data sets which did not show equal variances (indicated in the tables and legends) were analyzed performing *t* tests corrected for non-equal variances (Welch correction) offered by the Software GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Expression data of the reference gene (18S rRNA) were tested for differences between treatments by one-way ANOVA and Tukey's post hoc test using the GraphPad Prism software.

Results

RNA sequence database overview

RNA sequence data were generated from pooled cDNA samples of hemocytes, digestive gland, gill and siphon tissue of stress-treated *L. elliptica* individuals using high-throughput sequencing (GS FLX, Roche–454 Life Sciences). The 489,924 sequences generated at the ICMB, together with the 808,460 sequences provided by BAS, were assembled into 42,525 contigs (average size, 749 bp; N50, 877) and 114,322 singletons. The GC content of the contigs was 38.94 % (AT/GC ratio, 1.56). After BLAST annotation, 20,409 contigs were found to show high sequence similarity to previously published sequences from other organisms. Of the contigs, 618 were annotated by already published sequences from *L. elliptica*; 12,171 contigs contained conserved protein domains. An overview of the RNA sequence database annotation is given in Table 2.

Using key word searches, a high number of contigs with sequence similarity to known genes potentially involved in the *L. elliptica* stress defense and immune reactions were identified (Table 3, Supplementary Table S7). These included matches with high sequence similarity to functional components of the innate immune system such as the pro-inflammatory cytokine interleukin-17, members of the NADPH oxidase/dual oxidase family, cellular recognition receptors (Toll-like receptors), or antimicrobial peptides (theromacin, mytilin, and mytimycin). Further contigs with high sequence similarity to genes known to be involved in oxidative stress defense, general stress responses and oxygen sensing (HSPs, hypoxia-inducible factor, and prolyl 4-hydroxylase), detoxification (cytochrome P450), and autophagic processes (autophagy-related proteins) were identified. All these contigs are potentially involved in the maintenance of cellular homeostasis, and contigs with putative functions for oxidative stress defense (copper–zinc superoxide dismutase [Cu/Zn SOD] and catalase [Cat]), general stress

Table 1 Primer data for qRT-PCR analysis of selected candidate genes involved in the injury, immune system, and general stress responses (the contig ID of the *L. elliptica* RNA sequence database, reverse (R)

and forward (F) primer sequences, and amplicon length are given for each candidate gene)

Gene name	Primer name	Accession number	Amplicon length [bp]	Primer sequence
18S rRNA	<i>Le</i> -18S rRNA_F	HE804712	352	CACCACCAACCACCGAAT
	<i>Le</i> -18S rRNA_R			AAGACGAACGACAGCGAAAG
Actin	<i>Le</i> -Actin_F	HE804713	375	GCCAAATCCAGACGAAGG
	<i>Le</i> -Actin_R			TGAAGCCCAGAGTAAGAGAGG
Catalase	<i>Le</i> -Catalase_F	HE804714	378	CATTCTCTCCCGCACACAT
	<i>Le</i> -Catalase_R			GCCTTTCACGCTCCTCTG
Chitinase	<i>Le</i> -Chitinase_F	HE804715	223	TGCCAAAAGAAAATGCTGATT
	<i>Le</i> -Chitinase_R			GCCGTTGTTGAGTGTGCTAA
HSP70	<i>Le</i> -HSP70_F	HE804716	394	TCTCTCACCTCCAACCTTCTT
	<i>Le</i> -HSP70_R			TCCATCAAACACAATCTTCG
SOD [CuZn]	<i>Le</i> -SOD[CuZn]_F	HE804717	305	ACACCCAACGACTACCATCC
	<i>Le</i> -SOD[CuZn]_R			CGGCTACCACATCCACAAG
Theromacin	<i>Le</i> -Theromacin_F	HE804718	337	GCAGCCCACAGAGCAGTAAC
	<i>Le</i> -Theromacin_R			TAAAAGCGGACACCACCAT
TIMP	<i>Le</i> -TIMP_F	HE804719	322	CTGGGATTGCTTGCTGTGTA
	<i>Le</i> -TIMP_R			TCTGGGTCTGGATGAAAACA

response (HSP70), tissue regeneration and development (chitinase and actin), antimicrobial defense (theromacin), and response to injury and bacterial infection (tissue inhibitors of metalloproteinases [TIMP]) were selected for detailed investigation into gene expression changes related to starvation and injury treatments.

The RNA sequence database project was deposited as sequence read archive study ERP001323 and project number PRJEB33 at the European Nucleotide Archive (ENA). Assembled RNA sequences were deposited in the ENA as Transcriptome Shotgun Assembly, and accession numbers are given throughout the text or tables.

Effect of injury and starvation on hemocyte and siphon gene expression

Expression levels of candidate genes putatively involved in the immune and general stress responses of *L. elliptica* were

Table 2 Summary of *L. elliptica* contig annotation

	All sequences	100–500 bp	>500 bp
Total number of contigs	41,964	15,741	26,223
With BLAST matches	20,409	5,628	14,781
With assigned GO terms	8,934	1,885	7,049
With InterProScan matches	12,171	2,560	9,611
Without matches	20,985	9,932	11,053
% Annotated	49.99	36.90	57.85

investigated under injury and starvation in a size-dependent and time-dependent manner. Two tissues were analyzed: hemocytes, because they are major components of the immune system, and siphon, as it was directly inflicted with injury in the experiment.

The expression of the reference gene (18S rRNA) was not affected by treatment within the respective age groups. The most prominent effect on relative expression of selected candidate genes was observed in the hemocytes of the small individuals (Fig. 1, Supplementary Tables S1 and S3). After acute injury (2 days), expression levels of *Le*-TIMP, *Le*-SOD, *Le*-HSP70, *Le*-catalase, and *Le*-chitinase increased significantly (>10-fold) compared to non-injured individuals in the fed group. In contrast, small animals which were starved for 21 days prior to wound infliction did not respond with increased gene expression levels. A similar pattern of enhanced gene expression was observed in large injured individuals, but the response was less pronounced and induced levels did not reach significance. After 3 weeks of recovery and repair from the injury impact, gene expression levels were back to control levels in both size groups (Fig. 1).

Overall, feeding conditions had no significant effect on hemocyte gene expression in non-injured individuals. The response of different genes was, however, not uniform and minor trends were detectable. After the first 3 weeks of treatment, the majority of the genes under investigation appeared to be less highly expressed in fed animals compared with starved animals. This was especially the case for *Le*-TIMP, *Le*-SOD, *Le*-HSP70, and *Le*-catalase. After three

Table 3 (continued)

Category/gene name	Best blast hit UniProtKB/Swiss-Prot			Best blast hit NCBI, nt					
	<i>L. elliptica</i> contig accession	Accession	Description	Organism	E value	Accession	Description	Organism	E value
Hydramacin, theromacin	HE804743	Q9EPW9	Toll-like receptor 6	<i>Mus musculus</i>	3E-16	HM215599.1	Toll-like receptor 2	<i>Arvicola terrestris</i>	5E-12
Stress response	HE804718 ^a	B3RFR8	Hydramacin-1	<i>Hydra vulgaris</i>	4E-14	HM598084.1	Theromacin	<i>Hyrtopsis cumingii</i>	9E-29
Chaperonin	HE804744	Q64433	10-kDa HSP, mitochondrial	<i>Mus musculus</i>	5E-35	XM_003434292.1	Predicted: 10-kDa HSP, mitochondrial-like	<i>Canis lupus</i>	4E-41
DnaJ	HE804745	Q8WW22	Isoform 2 of DnaJ homolog subfamily A member	<i>Homo sapiens</i>	1E-137	NM_001079380.1	DnaJ (HSP40) homolog	<i>Xenopus (Siturana) tropicalis</i>	4E-139
HSP, low molecular weight	HE804746	P02518	HSP27	<i>Drosophila melanogaster</i>	2E-13	GQ384407.1	HSP22 isoform 1	<i>Venerupis philippinarum</i>	8E-96
HSP60	HE804747	P18687	60-kDa HSP, mitochondrial	<i>Cricetulus griseus</i>	0	FJ480412.1	HSP60	<i>Biomphalaria glabrata</i>	0
HSP70	HE804716 ^a	Q9UG39	Heat shock 70-kDa protein cognate 4	<i>Manduca sexta</i>	0	EF198332.1	HSP70	<i>Laternula elliptica</i>	0
HSP90	HE804748	O02705	HSP90-alpha	<i>Sus scrofa</i>	0	EU831278.1	HSP90	<i>Laternula elliptica</i>	0
Hypoxia inducible factor	HE804749	Q9YIB9	Hypoxia-inducible factor 1 alpha	<i>Gallus gallus</i>	2E-93	AB289857.1	Hypoxia-inducible factor 1 alpha	<i>Crassostrea gigas</i>	3E-111
Prolyl 4-hydroxylase	HE804750	Q98SW2	Hypoxia-inducible factor 1 alpha	<i>Oncorhynchus mykiss</i>	8E-04	AB289857.1	Hypoxia-inducible factor 1 alpha	<i>Crassostrea gigas</i>	5E-44
Thioredoxin	HE804751	P13674	Prolyl 4-hydroxylase subunit alpha-1	<i>Homo sapiens</i>	7E-89	XM_002429598.1	Prolyl 4-hydroxylase alpha-1 subunit precursor	<i>Pediculus humanus</i>	6E-108
Cellular homeostasis, detoxification, metabolism	HE804752	O96952	Thioredoxin	<i>Geodita cydonium</i>	1E-20	AY652616.1	Thioredoxin	<i>Chlamys farreri</i>	6E-27
Autophagy-related protein 2	HE804753	Q96BY7	Autophagy-related protein 2, homolog B	<i>Homo sapiens</i>	2E-52	XM_001489195.3	Predicted: ATG2 autophagy-related 2, homolog B	<i>Equus caballus</i>	2E-58
Autophagy-related protein 3	HE804754	Q9CPX6	Ubiquitin-like-conjugating enzyme ATG3	<i>Mus musculus</i>	5E-51	NM_001142489.1	Autophagy-related protein Aig-3-like protein	<i>Bombyx mori</i>	5E-59
Autophagy-related protein 4	HE804755	Q6PFS7	Ubiquitin-like-conjugating enzyme ATG3	<i>Danio rerio</i>	3E-61	XM_001601049.2	Predicted: autophagy-related 3-like	<i>Nasonia vitripennis</i>	2E-69
Autophagy-related protein 12	HE804756	Q6PZ02	Cysteine protease ATG4B	<i>Gallus gallus</i>	1E-110	AB513350.1	ATG4 mRNA for autophagy-related 4	<i>Haemaphysalis longicornis</i>	1E-121
Autophagy-related protein 12	HE804757	Q68FJ9	Cysteine protease ATG4D	<i>Xenopus laevis</i>	4E-31	XM_001181425.1	Predicted: similar to autophagy-related 4D	<i>Strongylocentrotus purpuratus</i>	7E-40
Cytochrome P450	HE804758	O94817	Ubiquitin-like protein ATG12	<i>Homo sapiens</i>	2E-31	XM_002939961.1	Predicted: autophagy-related protein 12-like (LOC100493403)	<i>Xenopus (Siturana) tropicalis</i>	7E-35
Ferritin	HE804759	Q9HCS2	Cytochrome P450 4F12	<i>Homo sapiens</i>	3E-85	HM126463.1	Cytochrome P450 family 4 protein	<i>Perinereis atubulitensis</i>	5E-109
Glutathione S-transferase	HE804760	Q92113	Cytochrome P450 family: steroid 17-alpha-hydroxylase/17,20 lyase	<i>Squalus acanthias</i>	8E-26	EF451959.1	Cytochrome P450-related protein	<i>Crassostrea gigas</i>	2E-38
Metallothionein	HE804761	P42577	Soma ferritin	<i>Lymnaea stagnalis</i>	3E-70	GQ139542.1	Ferritin-like protein	<i>Pinctada maxima</i>	7E-87
Housekeeping genes	HE804762	P19157	Glutathione S-transferase P1	<i>Mus musculus</i>	1E-48	EU131183.1	Pi class glutathione S-transferase	<i>Laternula elliptica</i>	0
Actin	HE804763	P80247	Metallothionein 10-II	<i>Mytilus edulis</i>	7E-27	DQ832722.1	Metallothionein 10a	<i>Laternula elliptica</i>	7E-42
18S rRNA	HE804713 ^a	P12716	Actin, cytoplasmic	<i>Pisaster ochraceus</i>	0	JN084197.1	Beta-actin	<i>Meretrix meretrix</i>	0
	HE804712 ^a	Q9Y4H2	Insulin receptor substrate 2	<i>Homo sapiens</i>	1E-02	AY192687.1	18S ribosomal RNA	<i>Laternula elliptica</i>	0

The information regarding the best BLAST match within the UniProtKB/Swiss-Prot and NCBI/nt-blast databases is given for each *L. elliptica* contig. Further contig details including sequence length and domain structure are listed in Supplementary Table S7

^a Genes/contigs investigated for differential expression in stress-treated *L. elliptica* individuals by qRT-PCR

additional weeks of food administration and starvation treatments, the reverse pattern was observed, with higher expression levels in fed individuals compared with starved individuals (Fig. 1). This was mainly due to a decrease in expression levels under the long-term mild starvation treatment, whereas gene expression levels in the food treatment remained constant throughout the experiment. Again, significant responses were predominantly and more often present in the group of smaller individuals, whereas larger individuals did only show smaller and less often responses.

In the siphon tissue, gene expression differed only slightly between the food treatment and injury groups (Fig. 2, Supplementary Tables S2 and S4). For *Le*-TIMP, *Le*-SOD, *Le*-HSP70, and *Le*-actin, a slight increase in expression levels after acute injury was observed in both size groups of starved and fed animals. In most cases, these changes failed to reach significance. In contrast to the results in hemocytes, “time” had a strong effect in siphon gene expression. In both size and food/starvation groups, expression levels increased between the first and second sampling dates in *Le*-TIMP, *Le*-SOD, *Le*-catalase, *Le*-chitinase, *Le*-HSP70, and *Le*-actin.

Effect of injury and starvation on the hepatosomatic index

Feeding conditions as well as injury infliction had no significant effect on the HSI in small individuals (Supplementary Tables S5 and S6). The HSI of large individuals was significantly affected by the feeding conditions. Non-injured animals had a lower HSI after 3 weeks of starvation conditions (median, 6.56), compared to non-injured animals after 3 weeks of food supply (median, 7.82; significantly different after Bonferroni post hoc test). The HSI in injured animals was not affected after either 3 or 6 weeks of starvation treatment (Supplementary Tables S5 and S6).

Discussion

Identification of genes involved in immunity

To enhance the amount of sequence information of genes involved in the stress response and immunity in bivalves, an RNA sequence database was generated from hemocyte and tissue samples of immune-stimulated *L. elliptica*. Half of the assembled contigs of *L. elliptica* could not be annotated using sequence similarity searching to known genes from other organisms or structurally characterized by InterProScan. This highlights the sparsity of sequence and functional information existing for marine bivalves and, in particular, *L. elliptica*. Nevertheless, the resulting contigs provide an excellent resource for the discovery of

putative genes involved in immunity and/or tissue repair in *L. elliptica* and will further help to identify such genes in other bivalves.

Components of the innate immune system identified in the *L. elliptica* database included cellular receptors, signal transduction molecules, effector proteins such as antimicrobial peptides, as well as components of detoxification, oxidative stress regulation, protein stabilization (HSPs), or cytoskeleton regeneration. These genes are important in the maintenance of homeostasis under stressful conditions and enable the animal to support an effective cellular response to environmental disturbance. Seven genes were selected from the database, which have previously been reported to play a role in the stress response, especially under injury, in other organisms (Montagnani et al. 2001; Lagente and Boichot 2010; Lee et al. 2011). These were used to conduct a functional analysis in the Antarctic bivalve *L. elliptica*.

Cross effects of starvation and injury on gene expression in hemocytes and siphon tissue

The expression of the selected antimicrobial and oxidative stress genes after injury in large individuals was not significantly influenced by the feeding conditions. The lack of change in gene expression under “starvation,” especially in large individuals, may be due to treatment time. In fact, *L. elliptica* seemed not to be critically starved, although they received no food for 3 weeks. This was substantiated by results in the young individuals. These animals have a higher metabolic activity and, although there was an overall reduction in stress gene expression during the experimental period, this was not significant. The HSI was not significantly affected in any treatment group of the small animals throughout the 3-week starvation experiment, whereas in large animals, the HSI was significantly reduced by 3 weeks of starvation (Bonferroni post hoc test). There were, however, no significant effects of starvation in all other treatments. In particular, HSI was also not reduced in injured animals after 3 weeks of starvation. Therefore, the reduced values in non-injured animals after 3 weeks of starvation may have been due to other, as yet unknown, factors and not solely due to starvation.

This lack of a response to 3 weeks starvation is almost certainly due to the adaptation of marine species to the cold Antarctic environment. Antarctic invertebrates such as the brachiopod *Liothyrella uva* were shown to have typically prolonged postprandial elevation of metabolic rates (Peck 1998, 2002; Peck and Veal 2001) which points to a long duration of food utilization and may delay the onset of severe starvation in Antarctic invertebrates. In addition, Antarctic invertebrates typically have low metabolic rates

as an adaptation to the extremely cold climate and intense seasonality with short summers and long periods of potential food shortage during winter (Clarke et al. 2008; Peck and Conway 2000; Schloss et al. 2012). The systemic occurrence of a starved phenotype may not be primarily apparent in the tissues tested in this experiment, especially the hemocytes, as one of the first biochemical symptoms would be mobilization of stored energy reserves, such as fat or protein. In addition, the induction of stress response genes in Antarctic invertebrates is potentially attenuated in Antarctic species compared with comparable temperate invertebrates (see the tiered response of heat shock genes to tidal emersion in Antarctic and Patagonian limpets in Pöhlmann et al. 2011).

In small animals, however, the gene transcription levels were generally higher in the hemocytes of fed specimens compared with starved specimens following injury. Cell counts conducted in the same small animals showed that the number of hemocytes per milliliter of hemolymph only increased in small injured animals under starvation (Husmann et al. 2011). Thus, whereas starved animals enhanced the number of hemocytes to mitigate the effects of injury, fed specimens may enhance the immunocompetence of the individual cells by inducing the expression of antistress and repair genes. This is in keeping with the idea that maintenance of immunocompetence is energetically costly as it involves gene transcription and protein synthesis (Lochmiller and Deerenberg 2000). The magnitude of the inducible response hence depends on the energetic reserves and feeding state of each individual. The origin of the additional free-floating hemocytes in starved and injured small bivalves is unclear.

Time-dependent patterns of gene expression in control animals

All selected candidate genes showed basal expression levels in the hemocytes and siphon tissue of non-injured animals. The downregulation of immune-related genes after 4 weeks starvation in non-injured *L. elliptica* may represent an adaptation to the decline of external stimuli in filtered seawater, as hemocytes respond directly to external stimuli such as bacteria. Increasing levels of most candidate genes in the siphon tissue of fed and starved individuals with time during the experiment possibly reflected a general adaptation to stress during prolonged maintenance in the holding system when they were not in their natural environment of being buried in sediment.

Changes of gene expression in the hemocytes of injured animals

As a first step towards investigating the molecular defense mechanisms in *L. elliptica* which might be influenced

by environmental stress in the WAP, putative candidate genes that are potentially regulated by food deprivation and injury were identified. The observation that injury had clearer effects on the upregulation of candidate genes compared with food-deprivation treatments in hemocytes highlighted the involvement of these genes in the immune defense of *L. elliptica*. In contrast, food deprivation had only minor effects on gene expression at least within the 4 weeks time frame, which may be too short to initiate starvation in Antarctic bivalves due to their low metabolic rates and their capacity to survive the intense seasonality of the Antarctic environment, as shown for other Antarctic marine invertebrates (Peck and Conway 2000; Peck 1998, 2002).

The most prominent effects were observed for *Le-TIMP*, *Le-SOD*, *Le-catalase*, *Le-chitinase*, and *Le-HSP70* in the hemocytes of small individuals following acute injury. In the hemocytes of large specimens and siphon tissue of both size groups, no strong effects were observed. This emphasizes the role of hemocytes as major players within the immune response in *L. elliptica* and corroborates our earlier findings of higher stress resistance in young individuals compared to old individuals (Husmann et al. 2011; Philipp et al. 2011; Clark et al. 2013). TIMP is an inducible protease inhibitor with a potential function in injury regeneration, remodeling of extracellular matrix, and defense reactions. In marine bivalves, TIMP-like molecules have been extensively investigated in the oyster *C. gigas* where they are most abundant in hemocytes. Their role in the stress response was illustrated by differential expression to bacterial challenge and injury. In *C. gigas*, TIMP-like molecules were shown to be upregulated in response to shell damage or infection with *Vibrio* spp. (Montagnani et al. 2001, 2007). However, De Decker and Saulnier (2011) as well as Labreuche et al. (2006) found strong downregulation or no response to *Vibrio* spp. challenge. The discrepancies in expression responses were attributed to the use of different *Vibrio* strains. Given the results presented here, *L. elliptica* *Le-TIMP* corroborates the role of an important effector in defense mechanisms and wound healing.

The upregulation of antioxidant enzymes such as SOD and Cat in injured animals suggested an increased need for cellular protection against increasing oxidative stress levels during acute injury. In bivalves, it is known that tissue lesions or invading bacteria trigger the “oxidative burst”-like generation of cytotoxic ROS including superoxide anions ($O_2^{\cdot-}$) and/or nitric oxides (Anderson 1994; Bettencourt et al. 2007; Husmann et al. 2011), which are produced during the phagocytosis of particles and invading pathogens (Adema et al. 1991). The observed increase in *Le-catalase* expression indicates enhanced intracellular H_2O_2 generation, which can activate further the immune response

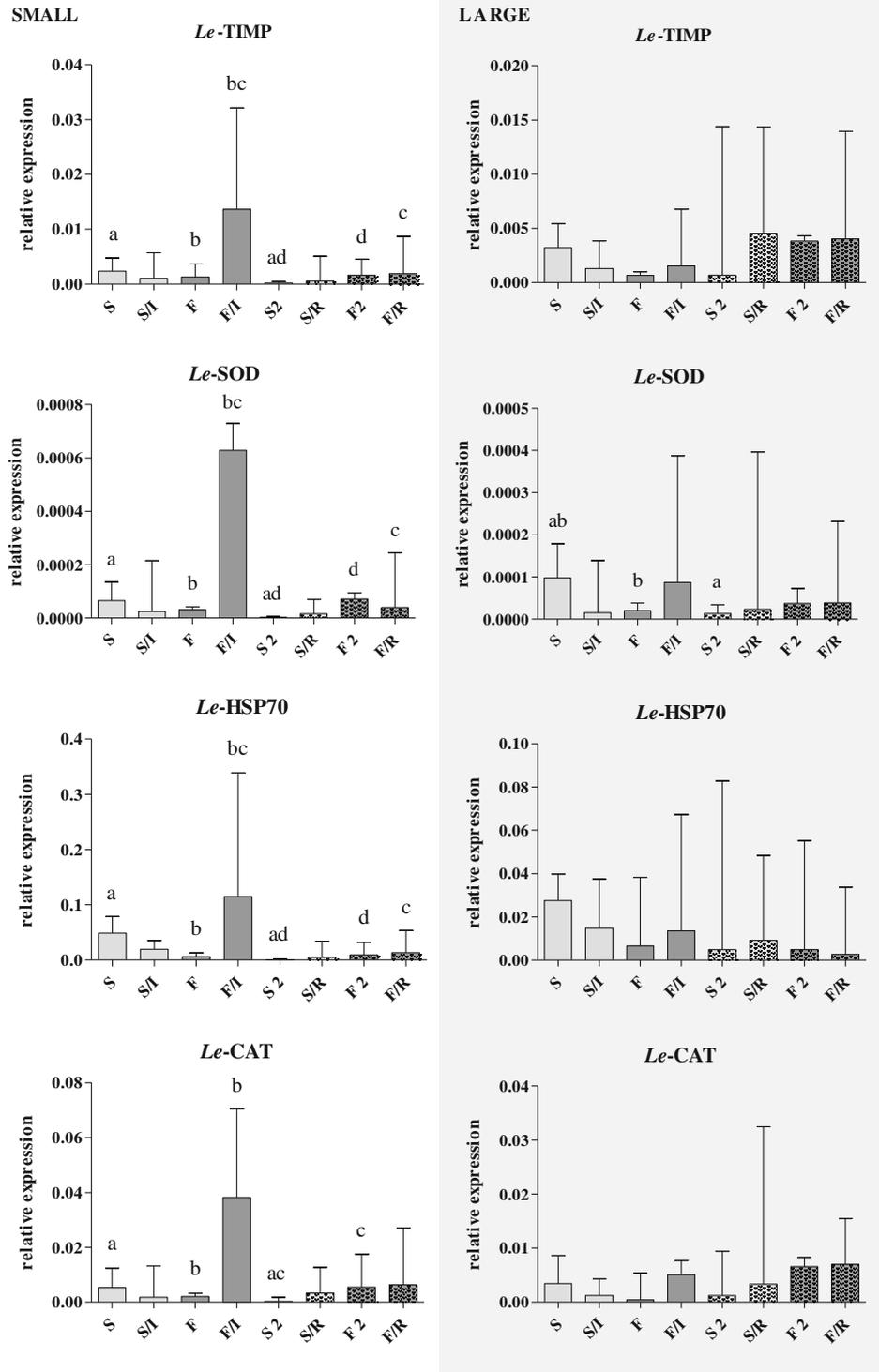
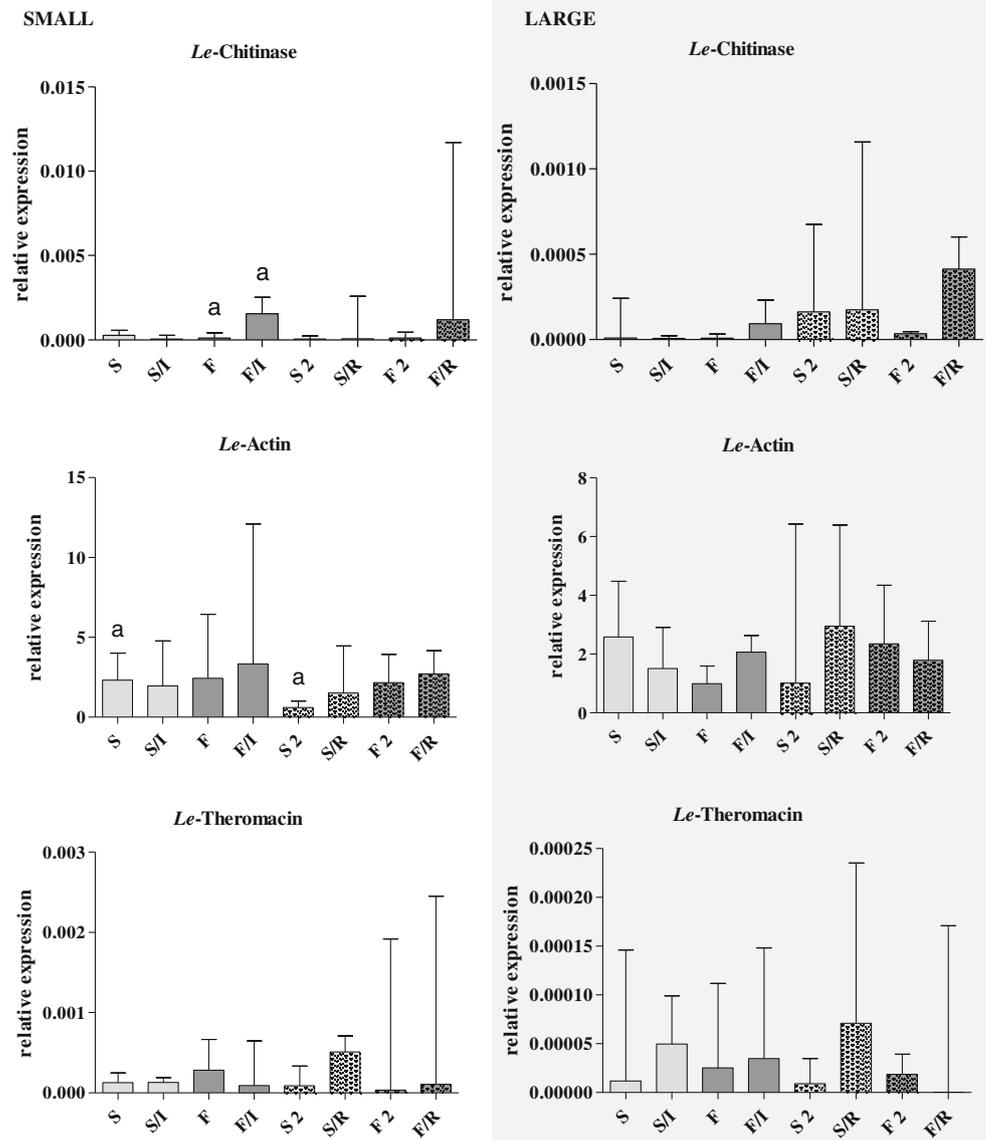


Fig. 1 *L. elliptica* hemocytes: Expression levels of selected contigs putatively involved in the stress and immune responses measured by qRT-PCR in different *L. elliptica* size/age groups individuals (small and large) sampled after 23 days of “food” (F) and “starvation” (S) treatment and experiencing acute (2 days after infliction) injury (S/I and F/I). Additional samples were taken after 44 days of “food” (F 2) and “starvation” (S 2) and recovering for 21 days from the previous inflicted injury (S/R and F/R). For the experimental details, see the

“Material and methods” section. Expression levels were normalized using the 18S rRNA as the reference gene. Data are presented as the median and interquartile range. $N=3-10$ individuals per group. Groups with similar letters are significantly different from each other with $p < 0.05$ (two-way ANOVA with Bonferroni post hoc test). Significant differences of *Le-SOD* expression levels between the groups of small individuals were identified using an unpaired *t* test with Welch correction for unequal variances or the nonparametric Mann–Whitney test

Fig. 1 (continued)



pathways involving NF κ B as found in the disk abalone *Haliotis discus discus* (De Zoysa et al. 2009; Ju et al. 2007).

Whereas some Antarctic marine invertebrates such as the sea star *Odontaster validus* or the gammarid *Paraceradocus gibber* lack a classical heat shock response (Clark et al. 2008b; Clark and Peck 2009), experimental work with *L. elliptica* indicated that the HSP70 genes are induced in response to heat stress (Clark et al. 2008a; Park et al. 2007), hypoxia (Clark and Peck 2009; Clark et al. 2013), and also changes in pH (Cummings et al. 2011). HSPs are often involved in stress defense and have been shown to be upregulated in hemocytes upon microbial challenge in bay scallops (*Argopecten irradians*) and zebra mussels (*Dreissena polymorpha*) (Xu and Faisal 2009; Song et al. 2006). Upregulation of *Le-HSP70* in injured *L. elliptica* is further consistent with the upregulation of SOD and Cat, as a response to physiological stress.

The upregulated expression of *Le-chitinase* in *L. elliptica* following injury is in line with findings in the oyster *C. gigas* (Badariotti et al. 2006, 2007a, b). mRNA levels of chitinases increased in the hemocytes of *C. gigas* following bacterial challenge and involvement in development, tissue growth, and remodeling was reported. In mammals, the functions of chitinases include regulation of immunity and apoptosis, stimulation of macrophages, as well as tissue remodeling and wound healing (Lee et al. 2011), and the expression level of chitinase 3-like protein 1 was upregulated in humans and mice following tissue damage (Bonneh-Barkay et al. 2010; Chen et al. 2004). These data suggest that, similarly, expression of chitinase-like genes in *L. elliptica* hemocytes may be involved in tissue repair or protection from bacterial infection following wound infliction.

The putative novel antimicrobial peptide *Le-theromacin* in *L. elliptica* was identified due to high sequence similarity

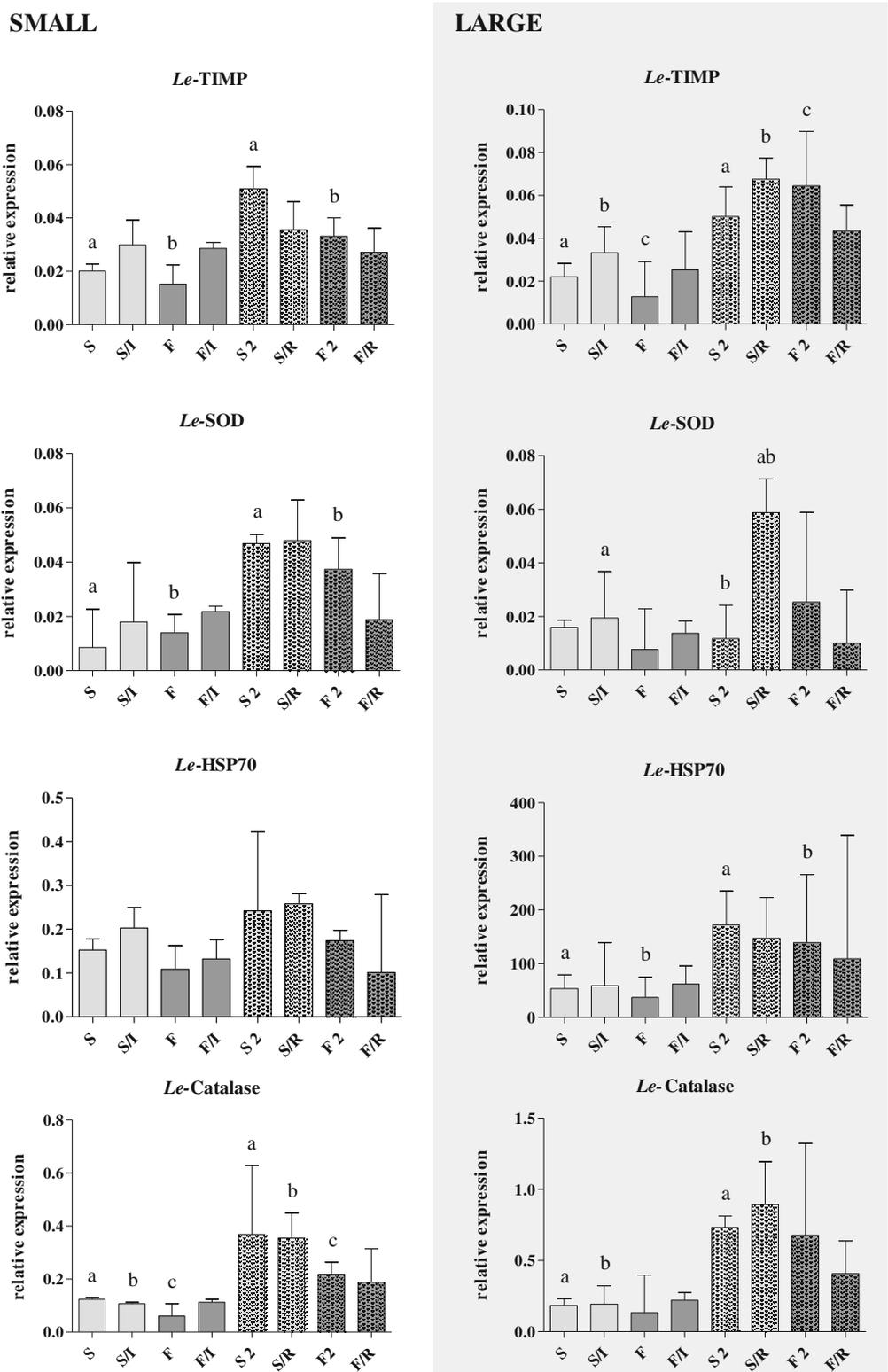
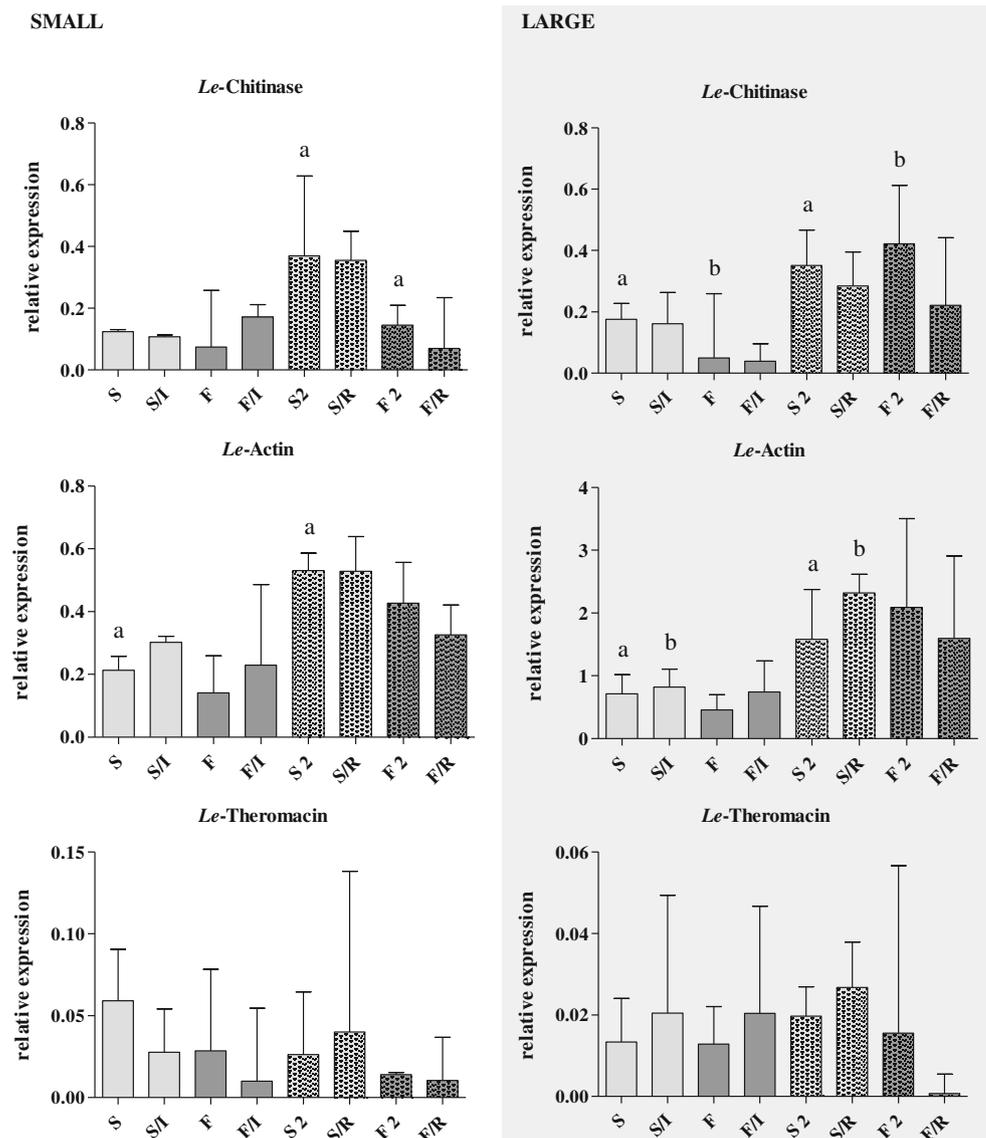


Fig. 2 Siphon gene expression levels measured by qRT-PCR in different *L. elliptica* size/age groups (small and large) sampled after 23 days of “food” (F) and “starvation” (S) treatment and experiencing acute (2 days since infliction) injury (S/I and F/I). Additional samples were taken after 44 days of “food” (F 2) and “starvation” (S 2) and recovering (S/R and F/R) from the previous inflicted injury

(for details, see the “Material and methods” section). Expression levels were normalized using the 18S rRNA as the reference gene. Data are presented as the median and interquartile ranges. $N=5-9$ individuals per group. Groups with similar letters are significantly different from each other with $p<0.05$ (two-way ANOVA with Bonferroni post hoc test)

Fig. 2 (continued)



to the theromacin sequence of the pearl mussel *Hyriopsis cumingii* (Xu et al. 2010). However, its function as an antimicrobial peptide in *L. elliptica* had yet to be confirmed. Expression of *Le-theromacin* was present in hemocytes and siphon tissues of untreated *L. elliptica*, but no induction upon injury was observed, indicating constitutive expression of this peptide. This is surprising, since antimicrobial peptides are major humoral effectors in the hemocyte immune response and may be induced by injury or bacterial challenge (Xu et al. 2010). The lack of response in *L. elliptica* may be due to several reasons. Sequence similarity does not necessarily equate to similarity of function (Clark et al. 2002) and, while *Le-theromacin* and *Hc-theromacin* share high sequence similarity, they may have divergent functions. If, however, this is an antimicrobial peptide, then the lack of response could be due to either not sampling at an appropriate time point or the experimental

stimulus was insufficient to induce upregulation of the gene.

Changes of gene expression in response to siphon injury

Injury to the siphon provoked only minor changes in gene expression in this tissue, although the siphons were visibly affected by the treatment. Older specimens, in particular, were observed to be sluggish, less mobile, and less active in filtration when injured. The overall upregulation of the selected candidate genes in siphon tissue 4 weeks following injury may reflect metabolic long-term changes to the conditions in the holding system without sediment, rather than an explicit response to injury. *Le-actin* expression was upregulated in the siphons of fed, starved, and injured animals after 4 weeks of treatment, possibly indicating investment into tissue maintenance. It is also possible that

the effect of injury on gene transcription is restricted to the injured area alone and the rest of the siphon tissue remains unaffected.

Differences in stress response between younger and older *L. elliptica*

The most pronounced differences in transcriptional expression were found in the younger animals, indicating that age is a significant factor in the transcriptomic defense response. This substantiates previous work in which hemocyte concentration and the intensity of the oxidative burst response under treatment with bacterial mimetics such as zymosan varied with age. Although small individuals had lower numbers of hemocytes per milliliter of hemolymph, the ROS generation in response to microbial stimulants was also more intensive compared with older individuals. Experimental treatments such as starvation and acute injury modulated the abundance of hemocytes, especially in older *L. elliptica* (Husmann et al. 2011). The more pronounced changes in gene expression in younger individuals seen in this study as a response to injury is in line with age-dependent physiological differences in marine bivalves (Philipp and Abele 2010) and especially in *L. elliptica* (Husmann et al. 2011; Philipp et al. 2005a, b). Also, the markedly higher susceptibility to injury in older *L. elliptica* individuals was in line with previous studies, which showed that their mortality rates following injury were higher, their respiration decreased under conditions of high turbidity, and their reburrowing ability was lower in old animals compared to younger specimens (Husmann et al. 2011; Morley et al. 2007; Peck et al. 2004; Philipp et al. 2011). The current gene expression studies investigating the combined effects of starvation and injury now also show that the induction period of stress genes in young animals is shorter compared with old injured animals. This might indicate a greater resilience and potentially a better physical condition, which was additionally corroborated by lower mortality rates found in all groups of younger individuals during the experimental treatment compared to older individuals. In summary, these data document the age-dependent stress response capacities in *L. elliptica*. The consequences of such lead to the prediction that, under conditions of environmental stress, e.g., ice scour and turbidity from increased glacial runoff, younger animals have a better chance of survival and recovery. This may lead to an alteration in the age structure of the general population towards a higher abundance of young (reproductively immature) animals. In the longer run, this will not only influence the composition and structure of *L. elliptica* populations but also the composition and function of Antarctic shallow benthic communities (Husmann et al. 2011; Philipp et al. 2011; Siciński et al. 2012).

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References

- Adema CM, Vandeutekomulder EC, Vanderknaap WPW, Meuleman EA, Sminia T (1991) Generation of oxygen radicals in hemocytes of the snail *Lymnaea stagnalis* in relation to the rate of phagocytosis. *Dev Comp Immunol* 15(1–2):17–26
- Ahn IY (1997) Feeding ecology of the Antarctic lamellibranch *Laternula elliptica* (Laternulidae) in Marian Cove vicinity, King George Island, during one austral summer. In: Battaglia B, Valencia J, Walton DWH (eds) Antarctic communities: species, structure and survival. Cambridge University Press, Cambridge, pp 142–151
- Anderson RS (1994) Hemocyte-derived reactive oxygen intermediate production in 4 bivalve mollusks. *Dev Comp Immunol* 18(2):89–96
- Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. *Aust Ecol* 26(1):32–46
- Atencio AG, Bertolin ML, Longhi L, Ferreyra GA, Ferrario ME, Schloss IR (2008) Spatial and temporal variability of chlorophyll-*a* and particulate organic matter in the sediments and the water column of Potter Cove (Antarctica). Reports on polar and marine research, vol 571. Alfred Wegener Institute for Polar and Marine Research, Bremerhaven
- Bachere E, Mialhe E, Noel D, Boulo V, Morvan A, Rodriguez J (1995) Knowledge and research prospects in marine mollusk and crustacean immunology. *Aquaculture* 132(1–2):17–32
- Badariotti F, Kypriotou M, Lelong C, Dubos MP, Renard E, Galera P, Favrel P (2006) The phylogenetically conserved molluscan chitinase-like protein 1 (Cg-Clp1), homologue of human HC-gp39, stimulates proliferation and regulates synthesis of extracellular matrix components of mammalian chondrocytes. *J Biol Chem* 40:29583–29596. doi:10.1074/jbc.M605687200
- Badariotti F, Lelong C, Dubos MP, Favrel P (2007a) Characterization of chitinase-like proteins (Cg-Clp1 and Cg-Clp2) involved in immune defence of the mollusc *Crassostrea gigas*. *FEBS J* 274(14):3646–3654. doi:10.1111/j.1742-4658.2007.05898.x
- Badariotti F, Thuau R, Lelong C, Dubos MP, Favrel P (2007b) Characterization of an atypical family 18 chitinase from the oyster *Crassostrea gigas*: evidence for a role in early development and immunity. *Dev Comp Immunol* 31(6):559–570. doi:10.1016/j.dci.2006.09.002
- Ballarin L, Pampanin DM, Marin MG (2003) Mechanical disturbance affects haemocyte functionality in the Venus clam *Chamelea gallina*. *Comp Biochem Physiol A* 136(3):631–640. doi:10.1016/S1095-6433(03)00216-2
- Barnes DKA, Conlan KE (2007) Disturbance, colonization and development of Antarctic benthic communities. *Philos T R Soc B* 362(1477):11–38. doi:10.1098/rstb.2006.1951
- Barnes DKA, Kaiser S (2007) Melting of polar icecaps: impact on marine biodiversity. *Encyclopedia of Life Support Systems*

- (EOLSS). Developed under the auspices of the UNESCO, EOLSS Publishers, Oxford, UK
- Barnes DKA, Souster T (2011) Reduced survival of Antarctic benthos linked to climate-induced iceberg scouring. *Nat Clim Chang* 1(7):365–368. doi:10.1038/Nclimate1232
- Beaz-Hidalgo R, Balboa S, Romalde JL, Figueras MJ (2010) Diversity and pathogenicity of *Vibrio* species in cultured bivalve molluscs. *Env Microbiol Rep* 2(1):34–43. doi:10.1111/j.1758-2229.2010.00135.x
- Bettencourt R, Roch P, Stefanni S, Rosa D, Colaco A, Santos RS (2007) Deep sea immunity: unveiling immune constituents from the hydrothermal vent mussel *Bathymodiolus azoricus*. *Mar Environ Res* 64:108–127
- Bonneh-Barkay D, Zagadailov P, Zou HC, Niyonkuru C, Figley M, Starkey A, Wang GJ, Bissel SJ, Wiley CA, Wagner AK (2010) YKL-40 expression in traumatic brain injury: an initial analysis. *J Neurotrauma* 27(7):1215–1223. doi:10.1089/neu.2010.1310
- Brown KM, Fraser KPP, Barnes DKA, Peck LS (2004) Links between the structure of an Antarctic shallow-water community and ice-scour frequency. *Oecologia* 141(1):121–129. doi:10.1007/s00442-004-1648-6
- Bussell JA, Gidman EA, Causton DR, Gwynn-Jones D, Malham SK, Jones MLM, Reynolds B, Seed R (2008) Changes in the immune response and metabolic fingerprint of the mussel, *Mytilus edulis* (Linnaeus) in response to lowered salinity and physical stress. *J Exp Mar Biol Ecol* 358(1):78–85. doi:10.1016/j.jembe.2008.01.018
- Butt D, Aladaileh S, O'Connor WA, Raftos DA (2007) Effect of starvation on biological factors related to immunological defence in the Sydney rock oyster (*Saccostrea glomerata*). *Aquaculture* 264(1–4):82–91. doi:10.1016/j.aquaculture.2006.12.031
- Canesi L, Gallo G, Gavioli M, Pruzzo C (2002) Bacteria–hemocyte interactions and phagocytosis in marine bivalves. *Microsc Res Techn* 57(6):469–476. doi:10.1002/Jemt.10100
- Canesi L, Barmo C, Fabbri R, Ciacci C, Vergani L, Roch P, Gallo G (2010) Effects of *Vibrio* challenge on digestive gland biomarkers and antioxidant gene expression in *Mytilus galloprovincialis*. *Comp Biochem Phys B* 152(3):399–406. doi:10.1016/j.cbpc.2010.06.008
- Chen L, Wu W, Dentchev T, Zeng Y, Wang JH, Tsui I, Tobias JW, Bennett J, Baldwin D, Dunaief JL (2004) Light damage induced changes in mouse retinal gene expression. *Exp Eye Res* 79(2):239–247. doi:10.1016/j.exer.2004.05.002
- Chu FLE, Volety AK, Hale RC, Huang YQ (2002) Cellular responses and disease expression in oysters (*Crassostrea virginica*) exposed to suspended field - contaminated sediments. *Mar Environ Res* 53(1):17–35
- Clark MS, Peck LS (2009) HSP70 heat shock proteins and environmental stress in Antarctic marine organisms: a mini-review. *Mar Genomics* 2(1):11–18. doi:10.1016/j.margen.2009.03.003
- Clark MS, Bendell L, Power DM, Warner S, Elgar G, Ingleton PM (2002) Calcitonin: characterisation and expression in a teleost fish, *Fugu rubripes*. *J Mol Endocrinol* 28(2):111–123. doi:10.1677/jme.0.0280111
- Clark MS, Fraser KPP, Peck LS (2008a) Antarctic marine molluscs do have an HSP70 heat shock response. *Cell Stress Chaperones* 13(1):39–49. doi:10.1007/s12192-008-0014-8
- Clark MS, Fraser KPP, Peck LS (2008b) Lack of an HSP70 heat shock response in two Antarctic marine invertebrates. *Polar Biol* 31(9):1059–1065. doi:10.1007/s00300-008-0447-7
- Clark MS, Thorne MAS, Vieira FA, Cardoso JCR, Power DM, Peck LS (2010) Insights into shell deposition in the Antarctic bivalve *Laternula elliptica*: gene discovery in the mantle transcriptome using 454 pyrosequencing. *Bmc Genomics* 11:362. doi:10.1186/1471-2164-11-362
- Clark MS, Husmann G, Thorne MAS, Burns G, Truebano M, Peck LS, Abele D, Philipp EER (2013) Hypoxia impacts large adults first: consequences in a warming world. *Glob Change Biol* doi:10.1111/gcb.12197
- Clarke A, Meredith MP, Wallace MI, Brandon MA, Thomas DN (2008) Seasonal and interannual variability in temperature, chlorophyll and macronutrients in northern Marguerite Bay, Antarctica. *Deep-Sea Res II Top Stud Oceanogr* 55(18–19):1988–2006
- Cook AJ, Fox AJ, Vaughan DG, Ferrigno JG (2005) Retreating glacier fronts on the Antarctic Peninsula over the past half-century. *Science* 308(5721):541–544. doi:10.1126/science.1104235
- Cummings V, Hewitt J, Van Rooyen A, Currie K, Beard S, Thrush S, Norkko J, Barr N, Heath P, Halliday NJ, Sedcole R, Gomez A, McGraw C, Metcalf V (2011) Ocean acidification at high latitudes: potential effects on functioning of the Antarctic bivalve *Laternula elliptica*. *PLoS One* 6(1):e16069. doi:10.1371/journal.pone.0016069
- De Decker S, Saulnier D (2011) Vibriosis induced by experimental cohabitation in *Crassostrea gigas*: evidence of early infection and down-expression of immune-related genes. *Fish Shellfish Immun* 30(2):691–699. doi:10.1016/j.fsi.2010.12.017
- de Eguileor M, Tettamanti G, Grimaldi A, Boselli A, Scari G, Valvassori R, Cooper EL, Lanzavecchia G (1999) Histopathological changes after induced injury in leeches. *J Invertebr Pathol* 74(1):14–28
- De Zoysa M, Whang I, Lee Y, Lee S, Lee JS, Lee J (2009) Transcriptional analysis of antioxidant and immune defense genes in disk abalone (*Haliotis discus discus*) during thermal, low-salinity and hypoxic stress. *Comp Biochem Phys B* 154(4):387–395. doi:10.1016/j.cbpb.2009.08.002
- Delaporte M, Soudant P, Moal J, Lambert C, Quere C, Miner P, Choquet G, Paillard C, Samain JF (2003) Effect of a mono-specific algal diet on immune functions in two bivalve species — *Crassostrea gigas* and *Ruditapes philippinarum*. *J Exp Biol* 206(17):3053–3064. doi:10.1242/Jeb.00518
- Delaporte M, Soudant P, Lambert C, Moal J, Pouvreau S, Samain JF (2006) Impact of food availability on energy storage and defense related hemocyte parameters of the Pacific oyster *Crassostrea gigas* during an experimental reproductive cycle. *Aquaculture* 254(1–4):571–582. doi:10.1016/j.aquaculture.2005.10.006
- Dixon P (2003) VEGAN, a package of R functions for community ecology. *J Veg Sci* 14(6):927–930
- Dominguez C, Eraso A (2007) Substantial changes happened during the last years in the icecap of King George, Insular Antarctica. *Karst and Cryokarst, Studies of the Faculty of Earth Sciences, University of Silesia* 45:87–110
- Donaghy L, Lambert C, Choi KS, Soudant P (2009) Hemocytes of the carpet shell clam (*Ruditapes decussatus*) and the Manila clam (*Ruditapes philippinarum*): current knowledge and future prospects. *Aquaculture* 297(1–4):10–24. doi:10.1016/j.aquaculture.2009.09.003
- Fisher WS, Auffret M, Balouet G (1987) Response of European flat oyster (*Ostrea edulis*) hemocytes to acute salinity and temperature changes. *Aquaculture* 67(1–2):179–190
- Hawley DM, Altizer SM (2011) Disease ecology meets ecological immunology: understanding the links between organismal immunity and infection dynamics in natural populations. *Funct Ecol* 25(1):48–60. doi:10.1111/j.1365-2435.2010.01753.x
- Hegaret H, Wikfors GH, Soudant P, Delaporte M, Alix JH, Smith BC, Dixon MS, Quere C, Le Coz JR, Paillard C, Moal J, Samain JF (2004) Immunological competence of eastern oysters, *Crassostrea virginica*, fed different microalgal diets and challenged with a temperature elevation. *Aquaculture* 234(1–4):541–560. doi:10.1016/j.aquaculture.2004.01.010
- Hunter S, Apweiler R, Attwood TK, Bairoch A, Bateman A, Binns D, Bork P, Das U, Daugherty L, Duquenne L, Finn RD, Gough J, Haft D, Hulo N, Kahn D, Kelly E, Laugraud A, Letunic I, Lonsdale D, Lopez R, Madera M, Maslen J, McAnulla C, McDowell J, Mistry J, Mitchell A, Mulder N, Natale D, Orengo C, Quinn AF, Selengut JD, Sigrist CJA, Thimma M, Thomas PD, Valentin F, Wilson D, Wu CH, Yeats C (2009) InterPro: the integrative protein signature database. *Nucleic Acids Res* 37: D211–D215. doi:10.1093/Nar/Gkn785

- Husmann G, Philipp EER, Rosenstiel P, Vazquez S, Abele D (2011) Immune response of the Antarctic bivalve *Laternula elliptica* to physical stress and microbial exposure. *J Exp Mar Biol Ecol* 398(1–2):83–90. doi:10.1016/j.jembe.2010.12.013
- Ju ZL, Wells MC, Heater SJ, Walter RB (2007) Multiple tissue gene expression analyses in Japanese medaka (*Oryzias latipes*) exposed to hypoxia. *Comp Biochem Phys C* 145(1):134–144. doi:10.1016/j.cbpc.2006.06.012
- Kadar E (2008) Haemocyte response associated with induction of shell regeneration in the deep-sea vent mussel *Bathymodiolus azoricus* (Bivalvia: Mytilidae). *J Exp Mar Biol Ecol* 362(2):71–78. doi:10.1016/j.jembe.2008.05.014
- Koutsogiannaki S, Kaloyianni M (2010) Signaling molecules involved in immune responses in mussels. *Invertebr Surviv J* 7:11–21
- Labreuche Y, Lambert C, Soudant P, Boulo V, Huvet A, Nicolas J-L (2006) Cellular and molecular hemocyte responses of the Pacific oyster, *Crassostrea gigas*, following bacterial infection with *Vibrio aestuarianus* strain 01/32. *Microbes Infect* 8(12–13):2715–2724
- Lagente V, Boichot E (2010) Role of matrix metalloproteinases in the inflammatory process of respiratory diseases. *J Mol Cell Cardiol* 48(3):440–444. doi:10.1016/j.yjmcc.2009.09.017
- Lee CG, Da Silva CA, Dela Cruz CS, Ahangari F, Ma B, Kang MJ, He CH, Takyar S, Elias JA (2011) Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. *Annu Rev Physiol* 73:479–501. doi:10.1146/annurev-physiol-012110-142250
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* 25(4):402–408. doi:10.1006/meth.2001.1262
- Lochmiller RL, Deerenberg C (2000) Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* 88(1):87–98
- Malham SK, Lacoste A, Gelebart F, Cuff A, Poulet SA (2003) Evidence for a direct link between stress and immunity in the mollusc *Haliotis tuberculata*. *J Exp Zool Part A* 295A(2):136–144. doi:10.1002/Jez.A.10222
- Momo F, Kowalke J, Schloss I, Mercuri G, Ferreyra G (2002) The role of *Laternula elliptica* in the energy budget of Potter Cove (King George Island, Antarctica). *Ecol Model* 155(1):43–51
- Monari M, Foschi J, Cortesi P, Rosmini R, Cattani O, Serrazanetti GP (2008) Chloramphenicol influence on antioxidant enzymes with preliminary approach on microsomal CYP1A immunopositive-protein in *Chamelea gallina*. *Chemosphere* 73(3):272–280. doi:10.1016/j.chemosphere.2008.06.033
- Montagnani C, Le Roux F, Berthe F, Escoubas JM (2001) Cg-TIMP, an inducible tissue inhibitor of metalloproteinase from the Pacific oyster *Crassostrea gigas* with a potential role in wound healing and defense mechanisms. *FEBS Lett* 500(1–2):64–70
- Montagnani C, Avarre JC, de Lorgeril J, Quiquand M, Boulo V, Escoubas JM (2007) First evidence of the activation of Cg-timp, an immune response component of pacific oysters, through a damage-associated molecular pattern pathway. *Dev Comp Immunol* 31(1):1–11. doi:10.1016/j.dci.2006.04.002
- Morley NJ (2010) Interactive effects of infectious diseases and pollution in aquatic molluscs. *Aquat Toxicol* 96(1):27–36. doi:10.1016/j.aquatox.2009.09.017
- Morley SA, Peck LS, Tan KS, Martin SM, Portner HO (2007) Slowest of the slow: latitudinal insensitivity of burrowing capacity in the bivalve *Laternula*. *Mar Biol* 151(5):1823–1830. doi:10.1007/s00227-007-0610-7
- Mydlarz LD, Jones LE, Harvell CD (2006) Innate immunity environmental drivers and disease ecology of marine and freshwater invertebrates. *Annu Rev Ecol Evol S* 37:251–288. doi:10.1146/annurev.ecolsys.37.091305.110103
- Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solyomos P, Stevens HHH, Wagner H (2011) vegan: community ecology package. R package version 2.0-0. Available at <http://CRAN.R-project.org/package=vegan>
- Oliver LM, Fisher WS, Winstead JT, Hemmer BL, Long ER (2001) Relationships between tissue contaminants and defense-related characteristics of oysters (*Crassostrea virginica*) from five Florida bays. *Aquat Toxicol* 55(3–4):203–222
- Ottaviani E, Franchini A, Malagoli D (2010) Inflammatory response in molluscs: cross-taxa and evolutionary considerations. *Curr Pharm Des* 16(38):4160–4165
- Oubella R, Maes P, Paillard C, Auffret M (1993) Experimentally induced variation in hemocyte density for *Ruditapes philippinarum* and *R. decussatus* (Mollusca, Bivalvia). *Dis Aquat Organ* 15(3):193–197
- Park H, Ahn IY, Lee HE (2007) Expression of heat shock protein 70 in the thermally stressed Antarctic clam *Laternula elliptica*. *Cell Stress Chaperones* 12(3):275–282
- Park H, Ahn IY, Lee JK, Shin SC, Lee J, Choy EJ (2009) Molecular cloning, characterization, and the response of manganese superoxide dismutase from the Antarctic bivalve *Laternula elliptica* to PCB exposure. *Fish Shellfish Immun* 27(3):522–528. doi:10.1016/j.fsi.2009.07.008
- Peck LS (1998) Feeding, metabolism and metabolic scope in Antarctic marine ectotherms. In: Pörtner HO, Playle R (eds) *Cold ocean physiology*. Cambridge University Press, Cambridge, pp 365–390
- Peck LS (2002) Ecophysiology of Antarctic marine ectotherms: limits to life. *Polar Biol* 25(1):31–40. doi:10.1007/s003000100308
- Peck LS, Conway LZ (2000) The myth of metabolic cold adaptation: oxygen consumption in stenothermal Antarctic bivalves. *Geol Soc Lond Spec Publ* 177(1):441–450. doi:10.1144/gsl.sp.2000.177.01.29
- Peck LS, Morley SA, Portner HO, Clark MS (2007) Thermal limits of burrowing capacity are linked to oxygen availability and size in the Antarctic clam *Laternula elliptica*. *Oecologia* 154(3):479–484. doi:10.1007/s00442-007-0858-0
- Peck LS, Veal R (2001) Feeding, metabolism and growth in the Antarctic limpet, *Nacella concinna* (Strebel 1908). *Mar Biol* 138(3):553–560. doi:10.1007/s002270000486
- Peck LS, Ansell AD, Webb KE, Hepburn L, Burrows M (2004) Movements and burrowing activity in the Antarctic bivalve molluscs *Laternula elliptica* and *Yoldia eightsi*. *Polar Biol* 27(6):357–367
- Philipp EER, Abele D (2010) Masters of longevity: lessons from long-lived bivalves—a mini-review. *Gerontology* 56(1):55–65. doi:10.1159/000221004
- Philipp E, Brey T, Pörtner HO, Abele D (2005a) Chronological and physiological ageing in a polar and a temperate mud clam. *Mech Ageing Dev* 126(5):598–609. doi:10.1016/j.mad.2004.12.003
- Philipp E, Pörtner HO, Abele D (2005b) Mitochondrial ageing of a polar and a temperate mud clam. *Mech Ageing Dev* 126(5):610–619. doi:10.1016/j.mad.2005.02.002
- Philipp E, Brey T, Voigt M, Abele D (2008) Growth and age of *Laternula elliptica* populations in Potter Cove, King-George Island. Reports on polar and marine research, vol 571. Alfred Wegener Institute for Polar and Marine Research, Bremerhaven
- Philipp E, Husmann G, Abele D (2011) The impact of sediment deposition and iceberg scour on the Antarctic soft shell clam *Laternula elliptica* at King George Island, Antarctica. *Antarct Sci* 23:127–138
- Philipp EER, Kraemer L, Mountfort D, Schilhabel M, Schreiber S, Rosenstiel P (2012a) The Transcriptome Analysis and Comparison Explorer—T-ACE: a platform-independent, graphical tool to process large RNAseq datasets of non-model organisms. *Bioinformatics* 28(6):777–783. doi:10.1093/bioinformatics/bts056
- Philipp EER, Lipinski S, Rast J, Rosenstiel P (2012b) Immune defense of marine invertebrates: the role of reactive oxygen and nitrogen species. In: Abele D, Vázquez-Medina JP, Zenteno-Savín T (eds)

- Oxidative stress in aquatic ecosystems. Blackwell, Chichester, p 524
- Pöhlmann K, Koenigstein S, Alter K, Abele D, Held C (2011) Heat-shock response and antioxidant defense during air exposure in Patagonian shallow-water limpets from different climatic habitats. *Cell Stress Chaperones* 16(6):621–632. doi:10.1007/s12192-011-0272-8
- Pruzzo C, Gallo G, Canesi L (2005) Persistence of vibrios in marine bivalves: the role of interactions with haemolymph components. *Environ Microbiol* 7(6):761–772. doi:10.1111/j.1462-2920.2005.00792.x
- Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* (Clifton, NJ) 132:365–386
- Santoro MG (2000) Heat shock factors and the control of the stress response. *Biochem Pharmacol* 59(1):55–63
- Schloss IR, Abele D, Moreau S, Demers S, Bers AV, González O, Ferreyra GA (2012) Response of phytoplankton dynamics to 19-year (1991–2009) climate trends in Potter Cove (Antarctica). *J Mar Syst* 92(1):53–66
- Siciński J, Pabis K, Jażdżewski K, Konopacka A, Błażewicz-Paszkowycz M (2012) Macrozoobenthos of two Antarctic glacial coves: a comparison with non-disturbed bottom areas. *Polar Biol* 35(3):355–367. doi:10.1007/s00300-011-1081-3
- Song LS, Wu LT, Ni DJ, Chang YQ, Xu W, Xing KZ (2006) The cDNA cloning and mRNA expression of heat shock protein 70 gene in the haemocytes of bay scallop (*Argopecten irradians*, Lamarck 1819) responding to bacteria challenge and naphthalin stress. *Fish Shellfish Immun* 21(4):335–345. doi:10.1016/j.fsi.2005.12.011
- Tirape A, Bacque C, Brizard R, Vandenbulcke F, Boulo V (2007) Expression of immune-related genes in the oyster *Crassostrea gigas* during ontogenesis. *Dev Comp Immunol* 31(9):859–873. doi:10.1016/j.dci.2007.01.005
- Tomanek L (2011) Environmental proteomics: changes in the proteome of marine organisms in response to environmental stress, pollutants, infection, symbiosis, and development. *Annu Rev Mar Sci* 3:373–399. doi:10.1146/annurev-marine-120709-142729
- Turner J, Bindschadler RA, Convey P, Di Prisco G, Fahrbach E, Gutt J, Hodgson DA, Mayewski PA, Summerhayes CP (eds) (2009) Antarctic climate change and the environment. Scientific Committee on Antarctic Research, Cambridge
- Venier P, Varotto L, Rosani U, Millino C, Celegato B, Bernante F, Lanfranchi G, Novoa B, Roch P, Figueras A, Pallavicini A (2011) Insights into the innate immunity of the Mediterranean mussel *Mytilus galloprovincialis*. *BMC Genomics* 12:69. doi:10.1186/1471-2164-12-69
- Xu W, Faisal M (2009) Identification of the molecules involved in zebra mussel (*Dreissena polymorpha*) hemocytes host defense. *Comp Biochem Phys B* 154(1):143–149. doi:10.1016/j.cbpb.2009.05.012
- Xu QQ, Wang GL, Yuan HW, Chai Y, Xiao ZL (2010) cDNA sequence and expression analysis of an antimicrobial peptide, theromacin, in the triangle-shell pearl mussel *Hyriopsis cumingii*. *Comp Biochem Phys B* 157(1):119–126. doi:10.1016/j.cbpb.2010.05.010