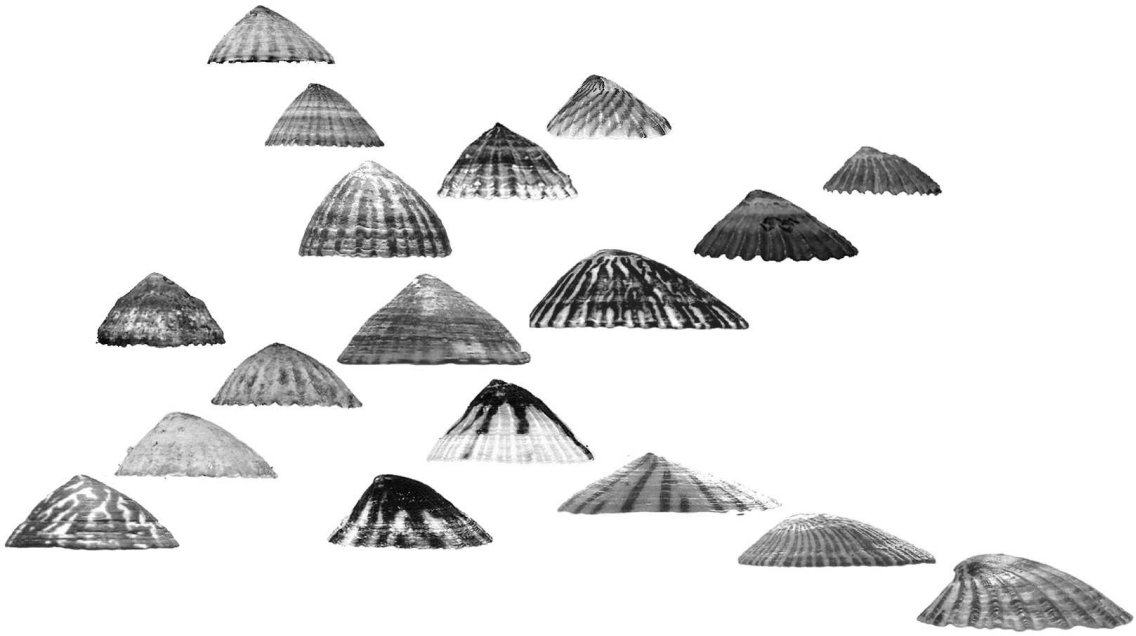
The Hsp70 Stress Response and its Genetic Background in South American intertidal and subtidal Limpets (*Nacella*)



Diploma degree thesis

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title illustration: arrangement of various South American *Nacella* shells
(from Valdovinos and Rüth (2005), De Aranzamendi et al. (2009) and www.conchology.be)

Contents

Non-standard abbreviations

bp	base pairs
C_T	threshold cycle
dNTP	deoxyribonucleotide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
F	fluorescence
Grp78	Glucose-regulated Protein (78kDa molecular chaperone)
HIF-1	Hypoxia-inducible Factor 1
Hsp70	Heat-Shock Protein (molecular chaperone of the 70kDa class)
mRNA	messenger ribonucleic acid
NTC	no-template control
PA	Punta Arenas (experimental site located in the Strait of Magellan)
PM	Puerto Montt (experimental site located in Northern Patagonia)
PCR	polymerase chain reaction
qPCR	quantitative (real-time) PCR
rpm	revolutions per minute
(r)RNA	(ribosomal) ribonuleic acid
RT	reverse transcription
T_m	melting temperature

Abstract

The alterations in environmental parameters anticipated during global climate change are predicted to intensify physiological stress to marine ectotherms and impose selective regimes on their stress-tolerance capacities. The limits to which the effects on ecosystems will be buffered by phenotypic plasticity and adaptation of populations appear unpredictable. One of the most universal reactions to environmental stress is the classic heat shock response, and its evaluation in intertidal invertebrates, which experience extreme fluctuations in stress levels, may be useful to advance in the mechanistic understanding of future regime shifts.

Using real-time RT-qPCR, I quantified the heat shock response in limpets of the genus *Nacella* that had been subjected to tidal emersion under natural conditions in field experiments at two locations in Chile.

In a subpolar limpet population from Punta Arenas in the Strait of Magellan, high-intertidal limpets showed delayed stress responses, including markedly lower expression of the *hsp70A* gene, in comparison to their subtidal congeners. Low-intertidal limpets from a warmer acclimatized population sampled at Puerto Montt (Central Chile) exhibited the highest stress response to tidal emersion, presumably due to higher temperatures affecting air exposed animals in a cold-temperate as compared to a subpolar environment. On the genomic level, the subtidal and intertidal subpopulations display a conspicuous divergence in two distinct *hsp70A* allele groups, as evidenced by a discrepancy in F'st estimates in comparison to neutral genetic markers.

My work indicates that Patagonian limpets show a graded heat shock response, increasing from South to North on a latitudinal gradient and from low to high on a tidal stress gradient. It provides indication for adaptive divergence of the *hsp70A* gene in these South American limpets, which might be explained by the selective effect of environmental stress caused by tidal emersion. Furthermore, it illustrates the great explanatory potential, but also conceptual difficulties of the heat shock response as an integrative biomarker for environmental stress. Stability assessment of reference genes is an integral part of quantification of the response, as stress conditions can cause down-regulation of constitutive genes and lead to overestimation of stress response levels. The quantification of response levels has to be performed against a thoroughly assessed background of inter-individual variation, population dynamic history and potential sequence adaptation.

1 Introduction

1.1 Heat shock on all scales: climate, change and stress

Global climate change will have omnipresent impacts on life on this planet and on the socio-economic development of humanity. Temperatures and other basic environmental parameters are predicted to change extraordinarily within the coming decades (IPCC 2007a), while extreme events like heat waves, droughts and floods will increase in frequency (Schär et al. 2004). This will not only affect humanity directly, but have a profound impact on the biotic resources that we depend on: Some plant and animal species that are vital for our nutrition may not be able to tolerate the changes and go extinct, others will change their geographic range and invade ecosystems that previously have not known them, leading to shifts and unpredictable collapses of biodiversity (Walther et al. 2002). Examples of ecological transformations in response to climate change are already accumulating rapidly, e.g. in the timing of spring events and the distribution ranges of species (Visser 2008, Parmesan 2006). In the light of the predictions of an accelerated warming, it has been estimated that one quarter of all species can be committed to extinction until the year 2050 (Thomas et al. 2004), and it is clear that some ecologically important species will be affected earlier than others (Hughes et al. 2003).
While the vulnerability of most species to climate change is unknown, how whole ecosystems will react to disruptions is a far more complex question. Especially, marine systems show dynamic nonlinear behaviour, and dramatic and unexpected regime shifts can be triggered by minor fluctuations (Hsieh et al. 2005). As poorly as the stability of ecosystems is understood, it is common ground that biodiversity on the planet level is a vital resource and a safeguard against potential disastrous corrosions of a functioning system (Reusch et al. 2005). To what extent will ecosystems on this planet be able to tolerate changes, and how much will they be impeded from doing this by exploitation and other anthropogenic impacts? (IPCC 2007b)

Change is nothing new to this planet's inhabitants. Fluctuations on the timescales of days, seasons or inter-annual cycles are known to every living thing. On the larger scale, the millennial climate oscillations by the glacial periods have played an important role in the distribution of species (Davis et al. 2005), and last but not least, thermal fluctuations in the earth's mantle produce the effects of plate tectonics (Schubert et al. 2001), forming the

framework for both terrestrial and marine habitats over geological times. These changes in space and time have produced barriers for dispersal, eliminated organisms that cannot adapt and created room to conquer for new ones (Hewitt 2001). Thus, change has shaped the diversity of life as we know it today. However, the rapid transformations predicted by climate models for certain regions of the world, might accelerate the rate of change in biodiversity in the near future (Reusch and Wood 2007). The key questions will be: How high is the level of flexibility that organisms have maintained as a result of their changeable history? To what extent can phenotypic plasticity and genetic adaptation of a population keep pace with new conditions?

Contrary to most other human impacts on the biota of this planet, a unique characteristic of climate change is that it has the potential to affect all organisms on a low level of organisation. Among the environmental factors expected to change are CO_2 concentration, temperature, pH value, UV radiation, and the resulting changes in ocean circulations and sea level rises (Harley et al. 2006). All these factors can cause physiological stress to the organisms and impose selection for stress-tolerance (Bijlsma and Loeschke 2005). To integrate these parameters into the expected response of species to stress is a major challenge across all biological levels. Seemingly subtle changes in physical and chemical parameters will influence the biochemistry, physiology and fitness of individual organisms, change the size and composition of populations, and finally re-model the genetic structure of a species, its distribution and species-species interactions, including those with humans. Considering the complexity of possible interactions, it appears impossible to disentangle the countless threads of cause and effect, go beyond correlative studies and create a mechanistic understanding of the evolutionary changes that can be expected (Vasemägi and Primmer 2005, Poertner and Farrell 2008). Model-systems that span a range of organisational levels can reduce internal complexity and are able to verify and quantify the stressful effects of environmental change on an organism, its population dynamics and the changes of regional distribution patterns. Then, we might be able to understand the large-scale changes that will happen around us and predict their future direction.

1.2 The heat shock response: physiology and genetics

Rising temperatures are expected to have the most immediate and fundamental effect of all the environmental parameters involved in climate change (Harley et al. 2006) and will cause physiological stress in all organisms, first of all in ectotherms. Temperature governs chemical flux rates and stability of macromolecules, thus all biological processes are dependent on the correct temperature window. Fluidity of cell membranes and enzyme activities are adjusted to the temperature in a habitat. For proteins to fulfil their functions in the cell, it is vital to maintain equilibrium between stability and lability in

their three-dimensional structure (Fields 2001). Thus, adaptation to an environmental temperature was an essential part of the evolutionary history not only of every organism, but also of every protein.

The most universal and best-investigated physiological reaction to thermal cellular stress is the classic heat shock response. It involves massive concerted production of different so-called heat shock proteins, among which the most abundant are the molecular chaperones of the Hsp70 class, formed by several related proteins of a molecular weight of 68-74 kDa (Lindquist 1986). High temperatures lead to an increase in denatured and misfolded proteins in the cell, which results in a loss of enzyme function as well as provoke the danger of toxic protein aggregation. When this accumulation exceeds a critical threshold, Hsp70s are synthesized in high amounts and help to stabilize and refold non-native proteins or mark them for degradation (Mayer and Bukau 2005). By stabilizing repair enzymes, they indirectly help to prevent persistent damage to other molecules, like chromosomal DNA.

Regulation of this response, as it is understood so far, involves a central heat shock transcription factor (HSF) that is actually bound to the Hsps. When proteins denature and expose hydrophobic patches, the Hsps bind these with high affinity and release the HSF, which in turn binds to typical short promoter sequences, the Heat Shock Elements (HSE), and induces strong up-regulations of HSPs. Although HSF1 universally activates transcription of all known heat shock genes and plays a role in thermal adaptation of organisms (Hofmann 1999), other regulative factors probably fine-tune the stress response, e.g. protein-protein interactions after binding and/or posttranscriptional regulation (Buckley et al. 2001). As manipulative studies in *Drosophila* have confirmed, an extremely high level of stress-induced Hsp70 would also be detrimental to cellular function, not only due to the high cost of synthesis in terms of energy and amino acids, but also because of the deleterious effects of the uncontrolled binding to cellular proteins (Krebs and Feder 1997b). Thus, expression levels of stress proteins are expected to be tightly regulated and evolutionarily adapted to the stress levels experienced by the organism.

Nevertheless, Hsp70s are not only synthesized in extreme stress events, but also perform basic physiological functions in protein folding and import into cellular compartments (Mayer and Bukau 2005). In evolution, their genes have undergone extensive duplication and sub-functionalisation and are generally divided into stress-inducible and constitutive (Hsc, heat shock cognate) isoforms, although in many cases it is not clear which is their function under natural conditions (Feder and Hofmann 1999, Boorstein et al. 1994). Hsp70 genes are present in all organisms so far investigated, from hyperthermophilic Archaea living at more than 100°C at deep-sea hydrothermal vents (Trent 1996) to cold-adapted Antarctic ectotherms (Clark and Peck 2009a). Putatively because of their high physiological significance and high structural restrictions, Hsp70s are among the most highly conserved of all proteins and have been used for basal phylogenetic studies (Feder and Hofmann 1999).

Because the heat shock response seems to be an almost universal stress reaction, it has been proposed as an integrative measure of the stress levels to which organisms are exposed under determined environmental conditions (Halpin et al. 2002, Tomanek 2010). The first step in establishing the heat shock response as a biomarker for climate change would be to assess short-term and long-term variation of Hsp expression among individuals of a population, in order to establish a baseline on which to build predictions about the effect of stress on the individual's performance, and thus its potential selective effect on the population, community and ecosystem levels (Lejeusne et al. 2006).

1.3 The intertidal habitat: stress gradients in two dimensions

Intertidal zones are a high-stress environment, in which stress-tolerance mechanisms can be studied on a small scale. Periodic tidal uncovering can cause abrupt temperature changes, desiccation and hypoxia, and in concert with UV radiation and physical disturbance from wave action and predators, can exert a stress scenario which imposes high energetic costs on its invertebrate inhabitants (Somero 2002). As many of these stressors are expected to increase in habitats influenced by climate change, intertidal habitats can serve as a natural laboratory for stress responses (Hofmann 1999).

From the point of view of biogeography, intertidal zones may be regarded as essentially one-dimensional: narrow corridors, continuous along the shores of the continents and with few potential barriers to gene flow other than mere distance (Sorte and Hofmann 2004). Accordingly, many intertidal organisms are found in an intermingling continuum of sister species, their thermal acclimatization varies with latitude, but they often show local adaptation by differences in morphology or predator evasion tactics (Hellberg 1998). On the other hand, one of the most obvious characters of intertidal habitats is vertical zonation: animals can be found in populations at typical height zones on rocky shores, often possess distinct morphological features, and their tolerance to tidal exposure stress seems to be fixed in the populations (Tomanek and Sanford 2003).

Numerous experimental studies of the heat shock response agree in the fact that induction thresholds are correlated with ambient temperature or stress levels normally experienced by the organisms (Feder and Hofmann 1999), and it has been suggested that habitual expression of Hsps may be part of the physiological strategy of intertidal organisms to occupy ecological niches close to their thermal limits (Tomanek 2010). Recent microarray studies of intertidal *Mytilus* mussels indicate that the tidal rhythm can force intertidal ectotherms into characteristic phases of fluctuation between functional groups of genes, corresponding to tidal emersion: the metabolism and cell-division phases, which are anti-correlated, and the stress-response after emersion (Gracey et al. 2008). From more than 4000 genes of many different functional classes, the authors found the Hsp70 genes to be most highly indicative of emersion stress and to closely follow body temperature of the

animals.

As two-dimensional gradients shape the stress conditions in the intertidal environment on a microhabitat scale, it might provide a model ecosystem for the more complicated changes that are to be expected in the three-dimensional marine environments. With regard to time as an additional dimension and the problem it poses for modelling of climate change effects, the thermal fluctuations in the intertidal might be a useful model to distinguish effects of extreme stress levels from effects of mean values. Therefore, an environment where life is dictated by perpetual change may be a good place to study global change.

1.4 The emersion experiment with *Nacella*

While laboratory studies are important in elucidating single or combined effects of stress parameters, field experiments are vital to confirm the significance of the results under natural conditions and determine the character of the response that can be expected under a complex combination of stressors.
The field experiment with limpets of the genus *Nacella* was designed to study the stress response to temperature elevation, air exposure and other stressors associated with tidal emersion. *Nacella* is distributed along the Pacific and Atlantic coastline of the Magellanic province in southern South America, where mostly rocky shores and a ragged topography with fjords, islands and channels create ample intertidal habitat for colonization and Molluscan fauna is diverse (Valdovinos et al. 2003, Crame 1997). *Nacella* shows a high degree of morphological variation in this region, and has been a battleground for taxonomists for decades (Valdovinos and Rüth 2005). They have been grouped into up to nine species according to morphological data, but recent molecular genetic analyses could separate only the two main groups, *N. magellanica* and *N. deaurata*, and show only very low genetic differentiation, which is suspected to be due to recent speciation (González-Wevar et al. 2010, De Aranzamendi et al. 2009). By real-time RT-qPCR, I assessed the differences in the expression of Hsp70 in the foot tissue of *Nacella* limpets, which were collected from intertidal and subtidal sites along the coastline of Southern Chile and subjected to air exposure experiments of varying duration. Additionally, I analysed the distribution of Hsp70 alleles among limpets, looking for possible differences between the two limpet groups. The results might provide an idea of the level on which adaptation to a stressful environment is taking place.

The aims of this work were

1. to quantify expression of the heat shock genes in experimentally treated *Nacella* limpets from Patagonia and compare the response between the three subpopulations,
2. to analyse allelic diversity of the heat shock protein genes in the experimental populations and contrast the variation on the phenotype and genotype levels.

2 Material and Methods

2.1 Sampling and experimental treatment

Limpets of the *Nacella magellanica/deaurata* species complex were collected from three different subpopulations in Southern Chile and subjected to *in situ* air exposure treatments of varying lengths, in order to simulate tidal emersion stress and quantify differences in their stress response. The limpets were sampled at two rocky intertidal locations in February 2009 (Fig. 2.1). At Chinquihue near Puerto Montt (PM) in Northern Patagonia, *N. magellanica* occurs at comparatively low individual density in the lower intertidal, where the animals experience a tidal range of approx. 6m and are exposed to air only during spring tides. At Bahia Laredo near Punta Arenas (PA) in the Strait of Magellan, the tidal range is only about 1.5m and *Nacella* occurs in two subpopulations of the species *N. magellanica* and *N. deaurata*. These are distinctive in shell morphology and preference of tidal position. The *N. magellanica* subpopulation in the upper intertidal undergoes daily tidal emersion of 2–4h (PA^{int}), while the *N. deaurata* group lives ca. 0.5m below the lowest low water line. Although it cannot be excluded that this subpopulation suffers occasional tidal effects by warming of the thin water cover, wave action or exposure at extreme weather events, we designated this subpopuation as subtidal (PA_{sub}).

All subpopulations were sampled at 8:30am, 1h (PM) and 2:30h (PA) before low spring tide, when water temperatures were 17°C and 9.5°C, respectively. Just before uncovering of the intertidal specimens, limpets were gently removed from the rocks with the help of a knife. For control values of gene expression, control animals were dissected immediately using a sterile scalpel blade and 100–150 mg samples of foot muscle tissue collected. For protection against RNA degradation, samples were stored in 1.5 ml of RNA*later* stabilization reagent (Qiagen) at ambient temperature for the rest of the day, and kept frozen at -20° until nucleic acid extraction.

For simulation of tidal air exposure, experimental specimens were placed in three separate dry plastic bowls on location (Fig. 2.2). After 2h, 6h and 12h, six animals were sampled from the treatments. Animals for the immersion control were kept in plastic bowls filled with 1l of fresh seawater for the same durations. Limpets from both treatments were dissected on location as described for untreated controls.

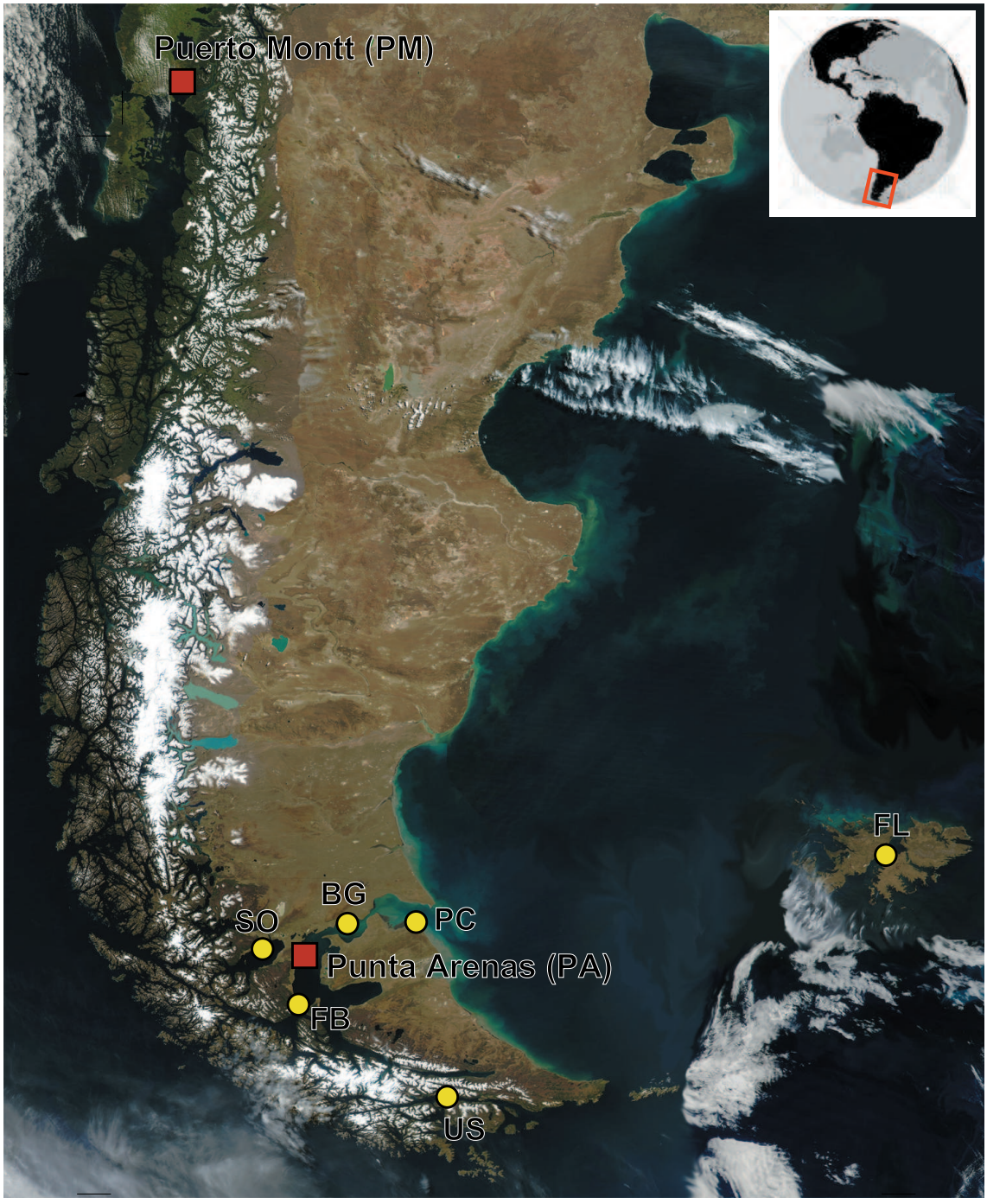

Figure 2.1: Satellite image of southern South America with sampling locations. Experimental sites (red squares) and other sampling locations (yellow circles) in Southern Chile and the Falkland Islands. Composite satellite image (NASA/MODIS).

Figure 2.2: *In situ* air exposure experiments with *Nacella*. Limpets were exposed to air for different durations and temperatures recorded (experiment at Puerto Montt, photograph by K.Poehlmann).

Natural fluctuation of air temperatures in the course of the experiment was measured with an electronic temperature sensor. Because of the different climatic conditions, animals at Puerto Montt experienced temperatures between 16° and 24°C in the course of the emersion experiment, while for both PA subpopulations, air temperatures were between 9° and 17°C (Fig. 2.3). For the PA subpopulations, experiments were conducted on two different days to ensure accurate sample handling under difficult weather conditions. The 12h experiment was conducted at air temperatures of 9–13°C, whereas temperatures on the following day, when the 2h and 6h experiments were carried out, were between 14° and 17°C.
Experiments and collection of tissue samples were performed by K. Poehlmann (AWI).

For sequence comparisons, genomic DNA samples were provided by K. Poehlmann from *Nacella* populations at the Chilean Pacific coast at Seno Otway and Ushuaia, from three populations in the Strait of Magellan, and from the Atlantic opening of the Strait at Punta Catalina (Tab. 2.1, Fig. 2.1). Additional RNA samples of the Antarctic congener *Nacella concinna* from St. George Island (South Shetland Islands, Antarctic peninsula) were provided by E. Weihe (AWI).

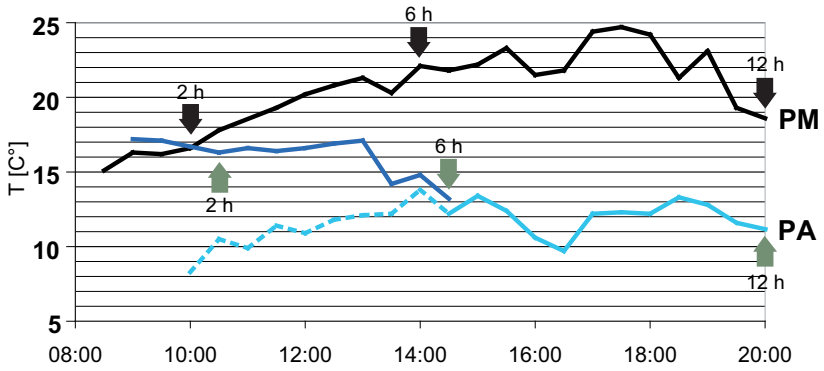

Figure 2.3: Air temperatures in the course of the emersion experiment in Puerto Montt (PM) and Punta Arenas (PA). Arrows mark time points of sampling, two curves are shown for Punta Arenas because 2h/6h treatments and the 12h treatment were conducted on two different days.

Table 2.1: Sampling locations of *Nacella* limpets in Southern Argentina and Southern Chile. Experiments were conducted at Puerto Montt and Punta Arenas, additional genomic DNA samples were collected at the other sites in the Magellanic Province and on the Falkland Islands.

Symbol	**Site**	**Coordinates**	**Sampled species**	**Zone**
PM	Puerto Montt	41°30'42"S 73°00'55"W	*N. magellanica*	low intertidal
PA int PA sub	Punta Arenas	52°56'56"S 70°47'44"W	*N. magellanica* *N. deaurata*	mid intertidal subtidal
SO	Seno Otway	53°03'47"S 71°17'37"W	*N. magellanica*	intertidal
US	Ushuaia	54°49'14"S 68°10'11"W	*N. magellanica* *N. deaurata*	intertidal subtidal
FB	Fuerte Bulnes	53°37'54"S 70°54'54"W	*N. magellanica* *N. deaurata*	mid intertidal subtidal
BG	Bahia Gregorio	52°36'12"S 70°11'02"W	*N. magellanica*	intertidal
PC	Punta Catalina	52°32'58"S 68°46'43"W	*N. magellanica*	intertidal
FL	Falkland Islands		*N. deaurata*	subtidal

2.2 Basic molecular genetic methods

2.2.1 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a standard method for specific *in vitro* amplification of DNA fragments. It utilizes a pair of short oligonucleotide primers flanking the DNA region to be amplified and a thermostable DNA polymerase in a temperature cycling protocol (Saiki et al. 1985). Denaturation at a temperature of 90–95°C leads to melting of the DNA double strand. In the annealing step, temperature is decreased to allow for hybridisation of the primers to the complementary region in the DNA strands. When temperature

is increased to the polymerase's activity optimum, it uses the 3'-OH ends of the primers to start synthesizing a DNA strand complementary to the target sequence (extension step). By cyclic repetition of these steps in a thermocycler, ideally an exponential amplification of the target DNA fragment occurs, which allows the detection of very small amounts of template DNA (Sambrook and Russell 2001).

For standard PCR amplification, HotMaster™ *Taq* DNA Polymerase (5 Prime GmbH) was used in the buffer provided by the manufacturer containing 2.5mM Mg^{2+}. The following ingredients were premixed in a master mix for each reaction:

H_2O	20.4 µl
Buffer (10x)	2.5 µl
Betaine (5 M)	0.3 µl
dNTP (10 mM)	0.5 µl
Primer (F/R) (100 µM)	0.1/0.1 µl
HotMaster Taq Pol. (5 U/µl)	0.1 µl

Concentration of DNA template solutions was measured in a NanoDrop™ ND-1000 Spectrophotometer (peqLab) and the templates diluted to approx. 10 ng/µl. After addition of 1 µl of template to each reaction, amplification was executed in a Mastercycler® gradient (Eppendorf) using the following program:

initial denaturation	94°C	2 min	
denaturation	94°C	20s	35x
annealing	50–54°C	20s	
extension	65°C	40s	
final extension	65°C	5 min	

Sizes of the PCR-amplified fragments were verified by standard agarose gel electrophoresis. 4 µl of PCR product were mixed with 1 µl Orange DNA Loading Dye (Fermentas Life Science) and run next to 2 µl of FastRuler™ Middle Range DNA Ladder (Fermentas) in TAE (Tris-Acetate-EDTA) buffer. Gels were stained with ethidium bromide and photographed under UV light in a Bio-Vision 3026 gel documentation system.

2.2.2 DNA sequencing

Dye-terminator sequencing is an enhancement of classic chain-termination Sanger DNA sequencing (Sanger et al. 1977) and still the standard method in automated sequencing, although next-generation methods are becoming increasingly economical and established. Its protocol is similar to a standard PCR (see 2.2.1), but only one primer is used and thus amplification is linear and not exponential. Included in the reaction mix are the four differently fluorescence-labeled dideoxy-NTPs, which act as terminators of the synthesis and create DNA fragments of varying lengths (Sambrook and Russell 2001). These

fragments are separated by size in an automated capillary electrophoresis and the DNA sequence of the template is read from the trace chromatogram produced by the order of the incorporated ddNTPs.

Prior to the sequencing reaction, excess primers and nucleotides were removed by digestion with ExoI nuclease and Shrimp Alkaline Phosphatase (Exo/SAPit! Kit, USB Corp./Affymetrix). The cycle sequencing reaction was conducted with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The following ingredients were used in each reaction, with all but the template DNA premixed in a 9.5 µl master mix:

H_2O	6 µl
5x Seq. buffer	1.5 µl
Primer F (10 µM)	1 µl
BigDye terminator	1 µl
cleaned PCR product	0.5 µl

Amplification was executed in a Mastercycler® gradient (Eppendorf) using the following program:

activation / initial denaturation	96°C	1 min	
denaturation	96°C	10s	25x
annealing	50°C	5s	
extension	60°C	4 min	

Unincorporated dye terminators were removed from the cycle sequencing product by gel-filtration in a DyeEx® 96 Kit plate (Qiagen) according to manufacturer's instructions. For separation of the DNA strands, cleaned samples were mixed with 10 µl HiDi™ Formamide (Applied Biosystems) and denaturated at 95°C for 3min. Samples were sequenced in an ABI*Prism* 3130xl capillary sequencer (Applied Biosystems) and chromatograms quality-checked in CodonCode Aligner V3.5.7 (CodonCode Corp.).

2.3 Primer design

For DNA sequence amplification by PCR and RT-qPCR, it is necessary to design short oligonucleotide primers that are complementary to the target sequence. This information can be obtained from sequences of closely related organisms or from alignments of conserved sequences available in public databases. When many polymorphic sites are present and for variable codon positions in reverse-translated amino acid sequences, degenerate primers can be used. These are actually mixtures of oligonucleotides that differ in sites where no certain sequence information of the target region is available. The selectivity of these primers can be influenced by the annealing temperature, primer concentration and annealing time, but often they lead to amplification of several loci. Therefore, the PCR

products are separated by molecular cloning.

2.3.1 Cloning of target genes

Degenerate primers reported by Clark and co-workers (Clark et al. 2008a) were used to amplify fragments of the target heat shock proteins and of β-actin from two *N. deaurata* and two *N. magellanica* samples in a standard PCR at an annealing temperature of 54°C. PCR products were run on an agarose gel and bands relating to the expected size of 400bp were cut out and eluted with a gel extraction kit (Eppendorf). Cleaned fragments were cloned with TOPO TA Cloning® Kit (Invitrogen) according to manufacturer's instructions: 13-15 ng insert were ligated with 10 ng pCR2.1-TOPO® vector, a vial of One Shot TOP10® Chemically Competent *E. coli* cells were transformed with 2 µl of the cloning reaction by heat shock. After incubation on selective LB plates over night, white colonies were picked and grown in SOC medium, and inserts were sequenced using the flanking vector primers provided by the kit's manufacturer.

2.3.2 Primers

DNA Sequences obtained from cloned fragments were aligned with ClustalW in CodonCode Aligner software V3.5.7 (CodonCode Corp.) and checked for conserved regions. 20–25 bp regions were chosen visually and with the help of Primer3 software (Rozen and Skaletsky 2000). Selected sequences were checked with Netprimer [www.premierbiosoft.com/netprimer] and all primers were designed to have a theoretical melting temperature between 55 and 58°C and low potential for formation of dimer and hairpin structures, as reflected by δG values no more negative than -8 kcal/mol.
qPCR-Primer sequences for the molecular chaperones of the HSP70-family (*hsp70A*, *hsp70B*, *hsc70* and *grp78*) were taken from the primers designed for *N. concinna* by Clark et al (Clark et al. 2008a). Primers for *histone H3* were designed to fit the sequence published for *N. deaurata* and *N. magellanica* by Nakano et al ((Nakano and Ozawa 2007), GenBank accession nos. AB433688 and AB433689). Primers for *HIF-1* (Hypoxia Inducible Factor 1) subunit α were designed to the sequence from *N. concinna* kindly supplied by Weihe et al (unpublished).

All oligonucleotide primers used for real-time RT-qPCR (tab. 2.2) and for standard PCR and sequencing (tab. 2.3) were synthesized commercially, purified from by-products and salts by Reverse Phase Cartridge absorption and quality controlled using mass spectroscopy (Biomers.net).

Table 2.2: Primers used for quantification in real-time qPCR. T_{ma} = average melting temperature of the amplified fragment (specific for the qPCR/melting conditions used, see 2.5.3)

Target	Primer	Sequence (5'-3')	Amplicon	T_{ma}
hsp70A	HSP70A-F	ATTCGATGACGAGACGGTTCA	110 bp	82.8°C
	HSP70A-R	AACGTCTTCAATTCGCTTTTGTA		
hsp70B	HSP70B-F	AGTTCACCGACGACACAGTAC	109 bp	82.7°C
	HSP70B-R	TATTTTAGTCTCTGATTTGTACTC		
hsc70	HSC70-F	AATTTGACGATGGACACGTTCAA	109 bp	82.3°C
	HSC70-R	GGTCTTTTGTTCACCCTTGTAG		
grp78	Grp78-F	CTTGGGATGATAAATCTGTCCA	108 bp	75.6°C
	Grp78-R	CTTTGTCAGAACCTTGTACATTA		
HIF-1 α	HIF-2F	CAGTACCGTTTTTTGGCTAAG	120 bp	79.9°C
	HIF-2R	GCTGAGAACATAGTGAACACAGAC		
β-actin	NAct-2F	CCTCACCGAACGTGGTTACT	106 bp	83.2°C
	NAct-2R	CATTTCCTGCTCGAAGTCG		
histone H3	H3-1F	CGGCGGCGTGAAAAAG	106 bp	85.1°C
	H3-1R	GAAGGGCAGCTTGCGGA		

2.4 RNA extraction

For measurement of gene expression in limpet foot muscle, total RNA was extracted from the samples using a commercially available chaotropic extraction reagent containing phenol and guanidine isothiocyanate, followed by liquid-phase separation with 1-Bromo-3-Chloropropane (Chomczynski and Mackey 1995). RNA is easily degraded by ubiquitous RNase A, which is produced in almost all animal cells as a defense against RNA viruses (Sambrook and Russell 2001), therefore, various precautions were taken in order to prevent RNA degradation. RNA extraction and isolation were performed under a fume hood in a separate working area. RNase-free plastic tubes and double-filter pipette tips (Eppendorf) were used, water and reagents were certified RNase-free, and pipettes, gloves, glass containers and all other re-used equipment was regularly sprayed with RNaseAWAY (Molecular BioProducts Inc.) and wiped off with Kimwipes (Kimberly-Clark). During homogenisation and until phase-separation, RNA is protected by the strongly denaturing effect of guanidine isothiocyanate on enzymes. During reverse transcription, RiboLock® RNase Inhibitor was added (Fermentas Life Science).

2.4.1 Tissue homogenisation and extraction

50–100 mg of each tissue sample were cut to small pieces of 1-3 mm diameter with a clean scalpel blade and transferred to a 2 ml tube with 1.4 mm and 2.8 mm ceramic beads (Precellys Keramik-Kit 1.4/2.8 mm, peqLab Biotechnologie) and 1.5 ml TRI reagent (Sigma-Aldrich). Tissues were homogenized by vigorous shaking in a three-dimensional

motion in a Precellys®24 Dual tissue homogenizer (Bertin Technologies) at 6500rpm for 25s.

After homogenisation, the tubes were centrifuged for 10 min (12,000g, 4°C) to separate cell debris, membranes and polysaccharides. For phase separation, 150 µl 1-Bromo-3-Chloropropane were added to the solution and the mix was vortexed for 15s, incubated for 15 min at room temperature and centrifuged for 15 min (12.000 g, 4°C). The aqueous upper phase was carefully removed and mixed with 750 µl Isopropanol, incubated for 10 min and centrifuged for 10 min (10,000g, 4°C) for precipitation of the RNA. The pellet was washed twice in ethanol and left to dry for up to 10 min. The dried pellet was resuspended in 50 µl 0.1mM EDTA in RNAse-free water (Fermentas Life Science) and dissolved by shaking at 55°C for 15 min in a heating block (Thermomixer, Eppendorf). RNA solutions were subsequently stored at -80°C.

2.4.2 RNA quantification and quality control

Concentration and purity of RNA solutions were assessed in a NanoDrop™ ND-1000 Spectrophotometer (peqLab). Absorbance at 260 nm was used to determine total nucleic acid concentration, assuming that an absorbance of 1 equals 40 ng/µl of single-stranded RNA (Sambrook and Russell 2001). Purity of RNA was judged by the absorbance ratio A_{260nm}/A_{280nm}, which should be between 1.8 und 2.1 for pure RNA, while lower values point to a contamination with proteins, DNA, phenol or other contaminants (Sambrook and Russell 2001, Engels and Lottspeich 2009). A random sample of 10 extracts was analysed for RNA integrity control by electrophoresis in an Bioanalyzer 2100 Expert (Agilent Technologies).

To prevent possible contamination with genomic DNA, the extracted RNA solutions were digested with RNase-free DNase (1 U per µg RNA) in a buffer containing $MgCl_2$ (Fermentas Life Science). In this buffer, the endonuclease cuts single- and doublestranded DNA randomly into mono- and oligonucleotides by hydrolyzing its phosphodiester bonds (Sambrook and Russell 2001). Following digestion at 37°C for 30 min, the DNase was inactivated by heating at 65°C for 10 min, after addition of EDTA to a slight excess over Mg^{2+} ions to prevent hydrolysis of RNA (Wiame et al. 2000).

2.5 RT-qPCR

Real-time RT-qPCR is a relatively newly established method for quantification of a specific RNA template (Gibson et al. 1996, Higuchi et al. 1992). It consists of (I) reverse transcription (RT) of the extracted mRNA to single-strand cDNA, and (II) quantification of the target cDNA template in real-time qPCR. The assay is very sensitive and accurate

over a wide quantity range, and thus being used in clinical analysis, biotechnology and microbial and functional ecology, but most parameters are still not standardized (Nolan et al. 2006, Ginzinger 2002). Comparability, reliability and reproducibility of publications are limited by the frequently incomplete documentation of the choices made for these parameters in the assay (Bustin et al. 2009). As a multi-step method, RT-qPCR is sensible to a variety of error sources. Assay reliability can be seriously reduced by inappropriate choices in sample handling, reaction parameters and data normalization (Huggett et al. 2005).

First of all, variances in sample collection and nucleic acid extraction can change the gene expression profile in the sample (Bustin et al. 2009). RNA integrity can have considerable impact on the stability of RT-qPCR quantity measurements (Fleige and Pfaffl 2006), mRNA appears to be differentially sensitive to degradation, and thus degradation could lead to high gene-specific variation and jeopardize calculation of expression results (Pérez-Novo et al. 2005). Generally it is agreed upon that moderately degraded RNA samples can be reliably analyzed when qPCR amplicons are short (<250 bp) and normalization against a reference gene is performed (Nolan et al. 2006, Fleige et al. 2006).

Reverse transcription is the step that introduces most of the experimental variation into RT-qPCR (Stahlberg et al. 2004a). The choice of primers used for reverse transcription is a matter of debate (Ginzinger 2002, Stahlberg et al. 2004b). Pools of gene-specific primers exhibit best efficiency, but have practical limitations, since the number of primers usable in an efficient, reproducible reaction is limited and additional genes can not be analysed later in the study without repeating the RT step and introducing additional technical variation (Nolan et al. 2006, Hunter et al. 2010). Random oligonucleotides can prime synthesis of all RNA species present, but cDNA yield often does not show linear correlation with RNA template amount (Bustin and Nolan 2004), probably due to variation in rRNA amount. By using oligo-dT primers, mRNA is principally copied from the 3'polyA-tail. Depending on the distance of the qPCR target sequence to the 3'end of the mRNA, an effective transcription is achieved when the extracted RNA is relatively intact and the RT protocol is adjusted to dissolve secondary structures (Nolan et al. 2006). Using a mix of oligo(dT)- and random primers might be a compromise between both strategies (Hunter et al. 2010).

The qPCR reaction can be inhibited by the reverse transcriptase, and by phenol or other compounds from the extraction process (Suslov and Steindler 2005), leading to lower C_T values and false estimates of amplification efficencies (Nolan et al. 2006). Therefore, the amount of RT product used as a template should be kept as small as possible using a very

sensitive polymerase, and samples can be cleaned before quantification. Contaminations of the tissue extract with genomic DNA that are carried over into the cDNA template solution can also lead to false postive measurements. To exclude this error source, qPCR primers can be designed to span introns, if sufficient genomic sequence information is available, or the RNA extract can be digested with DNase (Ginzinger 2002). In parallel with all samples, -RT (minus RT) controls should be analyzed, consisting of an RT reaction without the enzyme, to allow for detection of gDNA contaminations.

Normalization A variety of normalization strategies have been introduced to account for these uncertainties in the RT-qPCR protocol. Gene expression can be normalized to total RNA quantity by measurement of nucleic acid concentration in the extract, which corrects for variations in the extraction process - but since the RNA population of a cell predominantly consists of ribosomal RNA, imbalances in the mRNA/rRNA ratio can produce high variations (Vandesompele et al. 2002, Hendriks-Balk et al. 2007). Nevertheless, normalization of total RNA input amount into the RT reaction should be performed to ensure highly efficient and reproducible cDNA synthesis (Bustin et al. 2009, Nolan et al. 2006, Huggett et al. 2005, Stahlberg et al. 2004b). Quantification of a ribosomal transcript like 18S RNA in qPCR is also practiced, but when normalization of total RNA amount is performed, this will at best result in a circular statement.
Therefore, expression of the target genes is usually quantified in relation to one or more reference, internal control or 'housekeeping' genes, assuming that these do not vary in expression level. This can correct for many sources of error, most importantly differences in RNA concentration and integrity and variation of RT efficiency, because the control mRNA undergoes the same conditions as the target (Ginzinger 2002). Unfortunately, the idea of a universally stable 'housekeeper' is a historical carry-over from semi-quantitative times of Northern Blotting (Hendriks-Balk et al. 2007, Huggett et al. 2005). Several classic housekeepers have been demonstrated to be regulated under different experimental conditions and an alltime-stable gene probably doesn't exist (Vandesompele et al. 2002, Bustin and Nolan 2004). Nevertheless, the expression of several adequate reference genes, i.e. that are less variable than the target genes, can be averaged to increase statistical reliability (Andersen et al. 2004), using the geometrical mean because of the logarithmic scale of C_T values (Vandesompele et al. 2002, Pfaffl et al. 2004). On the other hand, choosing an inadequate reference gene can not only corrupt reliability of the data but lead to wrong conclusions if it is regulated under the investigated conditions (Huggett et al. 2005).
Two software programs have been used for estimation of stability of the candidate reference genes, GeNorm and NormFinder. GeNorm uses the standard deviation of the logarithmically transformed expressions of one gene relative to all other genes to calculate

the average pairwise variation M (Vandesompele et al. 2002). Highest-ranked genes are the most variable ones and are excluded from the calculation, until the two most stable genes are left. NormFinder uses a more complex model approach (Andersen et al. 2004) and allows calculation of inter-group gene variations between subgroups of, e.g., different treatments. The program checks for systematic variation from the average of all candidate genes, calculates a stability value for each single tested gene and also proposes a combination of two oppositely regulated genes that can be used as a theoretical normalization baseline.

2.5.1 Reverse Transcription

RNA was reverse transcribed into cDNA using Maxima™ Reverse Transcriptase (Fermentas Life Science) under high temperature conditions to mitigate variation caused by RNA secondary structures, and using an extended reaction time to approach 100% yield in reverse transcription. This enzyme is a thermostable modified version of the M-MuLV (Moloney Murine Leukemia Virus) RNA- and DNA-dependent polymerase and also possesses RNAse H activity, which degrades the RNA-strand in RNA/DNA heteroduplexes after reverse transcription.
6 µl of DNase-digested RNA solution (equals 1 µg of total RNA) were mixed with 50pmol of oligo-$(dT)_{18}$ primer, heated for 5 min at 65°C to dissolve RNA secondary structures and cooled on ice to allow for primer annealing. Subsequently, the master mix containing 5x RT buffer, dNTPs, RiboLock® RNase Inhibitor and 20U Maxima™ Reverse Transcriptase (all by Fermentas) were added, mixed gently and incubated for 40min at 55°C, followed by heat inactivation for 5min at 85°C. Templates were diluted 1:100 for subsequent measurements and stored at -80°C. In order to prevent physical degradation by freezing damage, no cDNA sample aliquot was frozen and thawed more than once.

2.5.2 Real-time quantitative PCR

Real-time qPCR is a variant of the standard polymerase chain reaction (see 2.2.1) that has been designed for quantification of the amplified fragment (Higuchi et al. 1992; 1993). Today's real-time cyclers are equipped with lights for excitation of a reporter dye that is included in the reaction mix, either covalently bound to a primer or reporter oligonucleotide (hydrolysis probes, molecular beacons, dual hybridization probes) or as a free dsDNA-binding dye (SYBR Green, EvaGreen). While reporter probes provide a specific signal and can be used for multiplex PCR of two targets in one reaction, unspecific dyes are cheaper, enable more flexible experimental design and amplification specificity can be confirmed by melt curve analysis (Kubista et al. 2006). During the reaction, the increase

in fluorescence emission is recorded in each cycle of the cycling protocol. Raw fluorescence data is plotted against cycle number in a sigmoidal curve that visualises the PCR amplification process, i.e. the increase in copies of the PCR target fragment. In the initial phase, increase in fluorescence is low and variable due to the stochastic effects of a relatively large amount of non-target molecules. After usually 7-15 cycles, depending on the input template amount, reaction conditions reach their optimum and amplification is nearly exponential. This phase, where the samples' amplification plots are linear and nearly parallel when depicted on a logarithmic scale ("log-linear phase"), is commonly used for quantification (Nolan et al. 2006, Ginzinger 2002). When large amounts of PCR product and pyrophosphate have accumulated, primers and nucleotides are nearing depletion and physical stress starts to affect the Taq polymerase, reaction efficiency decreases: the amplification plot passes its turning point and approaches a final plateau.

For readout of discrete values from real-time fluorescence plots and conversion to linear cDNA quantities, several methods are available. Most often, a threshold is defined, where fluorescence is substantially above background levels in all samples and in the log-linear phase of amplification. The cycle number in which this threshold is crossed by a sample's amplification curve is commonly defined as the C_T (threshold cycle) value (Gibson et al. 1996, Heid et al. 1996), or more generally termed C_q (quantification cycle) or CP (crossing point) (Bustin et al. 2009). Other methods try to derive equivalent values mathematically from the form of the single sample curve, e.g. Second Derivative Maximum (Cp) method, Sigmoidal Curve Fitting (SCF) and the Cy_0 method (Guescini et al. 2008).
The established standard is transformation of C_T values to linear concentrations by standard curve quantification (Kubista et al. 2006). A standard curve ($\log[\text{cDNA}]/C_T$) is generated for each primer pair by preparing a dilution series of an experimental cDNA sample (Pfaffl 2001). The slope of this straight line represents the amplification efficieny and should ideally be 1. Using this method, the linear range of the quantification assay can be tested and variations in primer efficiency can be compensated for. Other popular methods are ΔC_T and $\Delta\Delta C_T$ quantification, where identical reaction efficiencies are assumed and calculations are simplified by directly using C_T differences between treated and control animals, and to an internal control gene in the latter method (Livak and Schmittgen 2001). Efficiency-corrected versions of these methods have also been published (Pfaffl 2001, Schmittgen and Livak 2008), but their results are equivalent to using quantification with two standard curves.
Absolute quantification has not seen great success in RT-qPCR, as it requires substantially more experimental effort and reliability of the results is controversial. Since easily obtainable DNA standards cannot quantify the efficiency of reverse transcription, internal RNA standards have to be synthesized and evaluated (Bustin 2000).

qPCR was conducted using Type-It™ HRM PCR Master Mix with HotStarTaq® Plus DNA Polymerase and EvaGreen dye (Qiagen), 10pmol each of two standard oligonucleotide primers (table 2.2) were added. During the annealing/extension step of each PCR cycle, the dye was excited at a wavelength of 470nm and the flourescence measured at the emitting wavelength of 510nm. 0.4 µl of a 1:100 dilution of the reverse transcription product (equals approx. 20 ng cDNA) were added as a template to 10 µl reaction volume in transparent 0.1ml strip tubes (Qiagen). Cycling was performed in a Rotor-Gene® Q 5-Plex rotary cycler (Qiagen Corbett Life Sciences) using the following two-step cycling program:

activation / initial denaturation	95°C	5 min	
denaturation	95°C	10s	40x
annealing / extension	55°C	30s	
melt	65-90°C	0.3°C/2s	

In each run, one gene was quantified in 22 samples in triplicates, with two -RT controls and one NTC (no template control) to assess for DNA contaminations. To provide a stable normalization between runs, a double-strand cDNA sample was used as a calibrator for each batch, and controls and samples of the three treatments were distributed evenly between the runs to further reduce the risk of inter-run variations affecting the result.

2.5.3 Melt analysis

To confirm the specificity of the qPCR amplification, melt analysis was performed directly after the cycling. By increasing the temperature in increments of 0.3°C for 2s each, the DNA double strand is denaturated slowly and the nucleic acid dye released. The decrease in flourescence is recorded at each step and the derivative of the trace data ($\delta F/\delta T$) shows distinct melt peaks at the melting temperature T_m of the amplification products (Ririe et al. 1997). T_m of a DNA fragment depends mainly on its length and on its base composition, with a high GC content leading to a higher T_m because of the higher binding energy of the GC base couple. Reaction by-products like primer dimers and contaminating genomic DNA containing introns can be identified by their different T_m.
Replicates showing by-product peaks with a height of more than 10% of the main peak were discarded. In the initial phase of the experiment, size of the amplified fragments was confirmed by standard agarose gel electrophoresis (which could be performed without ethidium bromide staining, due to the UV-fluorescent properties of the EvaGreen dye used in the reaction). Two samples were sequenced for each product peak and queried by BLASTn in GenBank (Benson et al. 2008) to confirm identity of the measured fragment.

2.5.4 Raw data analysis, normalization and statistics

Real-time fluorescence trace values were recorded using the Rotor-Gene Q Series software V. 1.7.94 (Qiagen). For baseline correction of background fluorescence, the software options 'Dynamic Tube Normalization' and 'Slope Correction' were activated. The former calculates a 'takeoff' cycle of the reaction by the second derivative of the sample trace, and subtracts the background level during the amplification cycles prior to this cycle from the measured values. Slope correction normalizes gradual increases in background fluorescence between samples using a line-of-best-fit calculation. Although both of these corrections were applied, an effect on inter-sample differences was only observed in the early cycles of the log-linear phase and the threshold was set above this level.

For readout of raw data and conversion to linear template quantities, two parallel methods were applied and compared. Firstly, standard curve quantification of C_T values was used to test the linear range of the quantification assay and possible variations in primer efficiency. One sample of the 6h-treatment group was designated the standard sample, cleaned of inhibiting substances with spin columns (QIAquick PCR Purification Kit, Qiagen) and prepared into six consecutive 5-fold dilutions. For each primer pair, C_T values of the dilutions were measured and plotted against the log input concentrations, and the linear standard curve equation was calculated by the Rotor-Gene software:

$$C_T = M * log(c) + B$$

where c is the relative concentration of the sample, M is the slope, which relates to the efficiency of primer binding and amplification, and B is the intercept, which represents the takeoff cycle of the reaction (Fig. 2.4). The amplification factor, by which the template amount is multiplied each cycle is calculated as $a = 10^{(-1/M)}$ and should ideally be 2 (or $M = -3.322$). Amplification factors were slightly >2 in two cases, which is theoretically impossible and can be caused by the presence of PCR-inhibiting substances, e.g. the reverse transcriptase or phenol and other compounds from the extraction process (Suslov and Steindler 2005, Ginzinger 2002, Nolan et al. 2006).

All replicate C_T values were transferred to Excel 2002 (Microsoft Corp., USA), arithmetic means calculated and the linear expression value for each sample obtained using the rearranged standard curve equation:

$$c_s = 10^{\frac{C_{Ts}-B}{M}}$$

To quantify total cDNA amounts, $B = 1$ was set. In calculation of relative expression levels, the respective C_T value of the standard sample in each run was used as the intercept B to correct for possible variation of the reaction takeoff between runs.

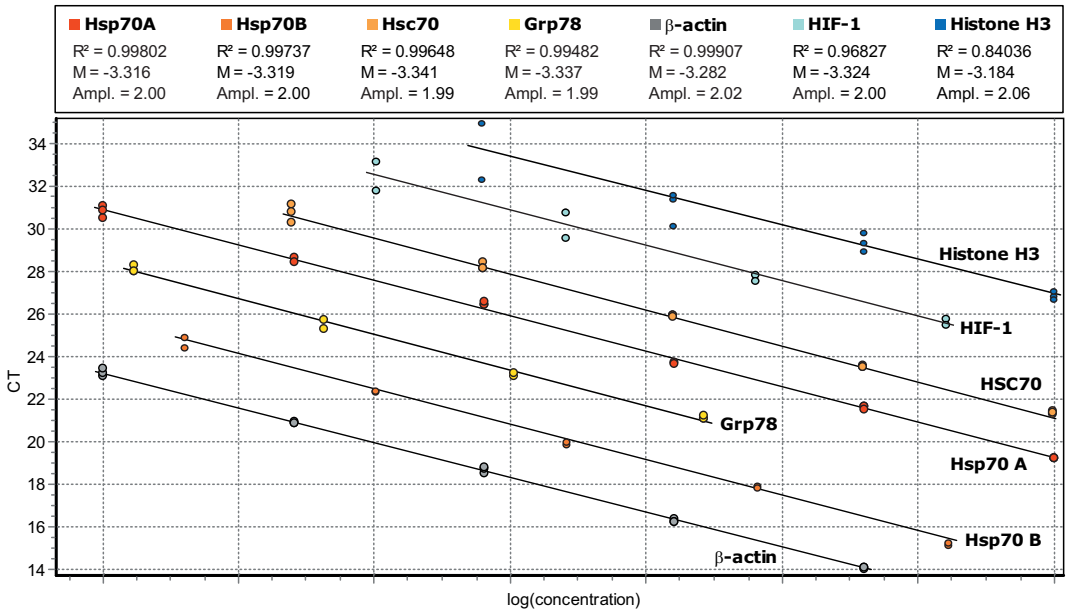

Figure 2.4: Standard curves for the quantified genes, showing the linear range of the assay. Correlation coefficient R^2 and slope M of the linear standard curves are given, all quantified samples were within the C_T range of the standard curve. Concentration values are in arbitrary units relative to the standard sample and curves have been moved on the x-axis for graphical clarity.

Values of treatment groups were checked for outliers using a modified Grubbs' test with $p \leq 0.01$ (Kaiser and Gottschalk 1972, Grubbs 1969) and, if present, one outlier per set removed. Stability of the candidate reference genes was tested by running the Excel macros GeNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004) on the expression data. Non-normalized data was tested for significant differences in expression levels between populations by one-way ANOVA with Bonferroni posttest using Graphpad Prism 5.01 (Graphpad Software Inc.). Relative expression data was normalized to reference genes by division by their geometric mean (Vandesompele et al. 2002), and tested for statistical significance of expression changes between the treatments by two-way ANOVA with Bonferroni posttest in Prism.

As an alternative approach for relative quantification, the Second Derivative Maximum method (sometimes called ΔC_p or CP method) was applied (Luu-The et al. 2005). This uses the shape of every single reaction fluorescence curve to calculate its takeoff point and amplification value. The takeoff point is defined as the cycle where the second derivative of the fluorescence plot is at 20% of the maximum level, and the amplification value is the average increase four cycles after the takeoff. Relative concentration of a sample is

calculated as

$$c_s = a_s^{C_{P0}-C_{Ps}}$$

where a_s is the amplification value of the sample, C_{P0} is the takeoff cycle of the control sample, and C_{Ps} is the takeoff cycle of the sample to be quantified (Pfaffl et al. 2002).

Amplification and takeoff values were output by the 'Comparative Quantification' feature in the Rotor-Gene Software. Replicates with amplification values under 1.5 or above 2.3 were discarded and sample averages transferred to REST 2009 software (Pfaffl et al. 2002) in 'RG mode', which calculates the relative up- or down-regulations and tests for significance by bootstrapping over treated samples and untreated controls (2000 iterations).

2.6 Analysis of HSP70 allelic diversity

2.6.1 Sequencing of HSP70 alleles

The heat shock protein genes *hsp70A*, *hsp70B* and *hsc70* were sequenced from genomic DNA and cDNA samples using a specific forward primer and a degenerated reverse primer (Tab. 2.3) in a standard PCR as described in 2.2.1, but without the addition of betaine, using 0.25 µl of reverse primer and changing the cycle protocol to an annealing time of 30s and an extension time of 2:30 min. The obtained fragments had a size of 1200bp. *Hsp70A* and *hsp70B* were sequenced in forward direction using a second specific forward primer, while *hsc70* was sequenced using the same forward primer, yielding approx. 800bp of good-quality sequence. For comparison with the congener *N. concinna*, *hsp70A* and *hsp70B* in four cDNA samples from *N. magellanica* in Puerto Montt and *N. deaurata* in Punta Arenas were also sequenced in reverse direction, yielding a 1000bp fragment. Sequences were visually examined in CodonCode Aligner (CodonCode Corp.). Mutations were automatically called by the software and checked by eye. Sites were categorized as ambiguities when a second peak of the same height was present, or when at least half of the height of the main peak was reached and the main peak was considerably lower than neighbouring bases. For confirmation of the ambiguities used as heterozygous sites in the *hsp70A* allele analysis, six fragments were additionally sequenced in reverse direction using a specific reverse primer (Tab. 2.3).

2.6.2 Phylogenetic analyses

Heat shock protein *hsp70A*, *hsp70B* and *hsc70* gene sequences from Patagonian and Antarctic *Nacella* were used to derive a phylogeny of the three heat shock proteins. Forward and reverse sequences were aligned using ClustalW in CodeAligner and exported

Table 2.3: Primers used for standard PCR and sequencing of HSP70 alleles. One specific forward and one degenerate reverse primer were used in PCR to produce amplicons of the Hsp genes, and specific primers used in the cycle sequencing reaction.

Target	Primer	Sequence (5'-3')
hsp70A	HSP70A-1F	AAGTTATGTGGCGTTCACAGAC
	HSP70A-2F	TTTGAATCCATCTAACACCATCTTC
	HSP70A-3R	AACCCCCAACCAGAACAAC
hsp70B	HSP70B-1F	ACCTAGCTATGTAGCTTTCACTGAT
	HSP70B-2F	ATTAAATCCATCGAATACTGTGTTT
	HSP70B-3R	TTGATAGATTTGTTTAGCTCCCTG
hsc70	HSC70-F	AATTTGACGATGGACACGTTCAA
hsp70A+B+hsc70	degHSP70-1R	GCNACAGCYTCRTCNGGRTT

to MEGA 4 (Tamura et al 2007). A phylogenetic tree was constructed using the maximum parsimony method on all nucleotide sites, and tested by bootstrapping over 1000 replications.

hsp70A sequences from genomic samples were collapsed to haplotypes with Fabox 1.35 (Villesen 2007), and used to construct a statistical parsimony haplotype network with TCS 1.21 (Clement et al. 2000). Tests for linkage disequilibrium of *hsp70A* and *hsp70B* were conducted with Genepop 4.0 (Rousset 2008). The *hsp70A* locus was tested for heterozygote deficiency and excess by a score test using the complete enumeration algorithm as implemented in the software. Estimates of F_{st} between subpopulations were computed using Arlequin 3.5.1.2 (Excoffier et al. 2005). For comparison with genetic distance, additional pairwise F_{st} values from eight microsatellite loci identified in Magellanic *Nacella* were used (Poehlmann and Held, in prep.).

3 Results

3.1 Limpet shell sizes

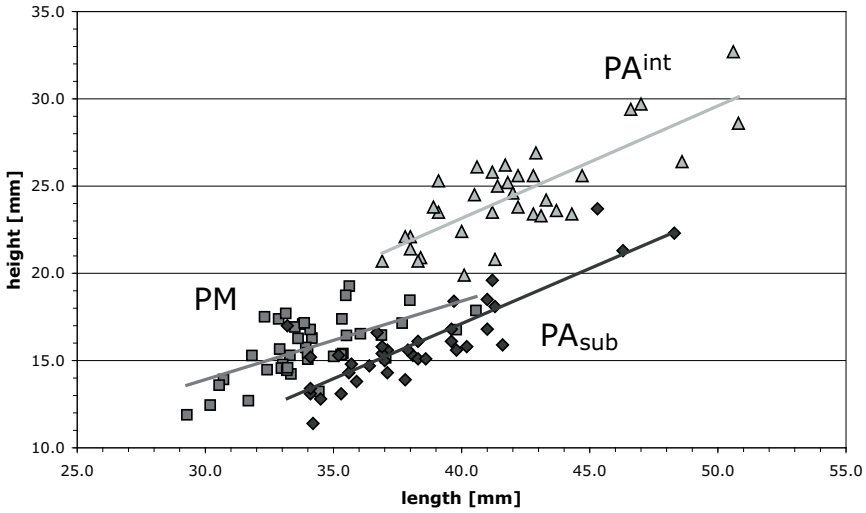

Figure 3.1: Limpet shell sizes in the three subpopulations. Shell height and shell length of experimental specimens from the lower littoral in Puerto Montt (PM, squares), from the upper intertidal zone in Punta Arenas (PA^{int}, triangles) and from the subtidal zone in Punta Arenas (PA_{sub}, diamonds), with linear regression curves.

Shells of intertidal *N. magellanica* sampled at Punta Arenas were significantly larger than subtidal *N. deaurata*, while subtidal animals had a higher shell height to length ratio, as measured with an electronic calliper (Fig. 3.1). Limpets from Puerto Montt were intermediate in height-length ratio and generally of smaller sizes than both Punta Arenas subpopulations.

3.2 Gene expression

3.2.1 qPCR amplicon identity

Direct sequencing of the qPCR products for each distinctive melt peak and BLASTn searches in GenBank confirmed relation of the amplified fragments to the expected genes

(Tab. 3.1).

Table 3.1: Characteristics of the fragments quantified in qPCR. Target genes, amplicon length (without primer sequence), amplicon melting point (specific for reaction conditions), and the three top-scoring hits in GenBank by blastn search with standard parameters (2010-09-04) for the consensus sequence of three sequenced samples.

target	amplicon		GenBank hits (blastn)		
	length [bp]	Tm [°C]	Accession no.	Description (abbr.)	Identities
hsp70A	110	82.8	AM293594	*Nacella concinna* HSP70A mRNA	71/72
			AY291333	*Blomia tropicalis* Mag29 allergen mRNA	53/65
			AY227751	*Trichinella spiralis* heat shock protein 70 gene	54/70
hsp70B	109	82.7	AM293595	*Nacella concinna* HSP70B mRNA	64/65
			XM2733657	*Saccoglossus kowalevskii* HSC71 mRNA	51/58
			DQ062090	*Lamtostyla sp.* LPJ-2005 HSP70 mRNA	52/60
hsc70	109	82.3	AM293597	*Nacella concinna* HSC70 mRNA	62/65
			EU977182	*Tetranychus cinnabarinus* HSP70-4 mRNA	49/61
			AJ318883	*Ostrea edulis* hsp70 gene	46/57
grp78	108	75.6	AM293596	*Nacella concinna* GRP78 mRNA	63/63
			XM2109793	*Trichoplax adhaerens* hypothetical protein, mRNA	46/58
			AB122065	*Crassostrea gigas* GRP78 mRNA	36/44
β-actin	106	83.2	DQ787858	*Mizuhopecten yessoensis* beta-actin mRNA	63/67
			AB071191	*Crassostrea gigas* actin 2 mRNA	63/67
			U55046	*Placopecten magellanicus* actin mRNA	63/67
HIF-1	120	79.9	AB500182	*Homo sapiens* HIF1A mRNA	37/47
			AY455802	*Canis familiaris* HIF-1 alpha subunit mRNA	37/47
			DQ838046	*Bos grunniens* HIF isoform 1a mRNA	38/49
H3a	106	85.1	AB433689	*Nacella magellanica* histone H3 gene	63/63
			AB433688	*Nacella deaurata* histone H3 gene	63/63
			HM162503	*Polycera* cf. *capensis* histone H3 gene	59/63

3.2.2 Total gene expression levels

Results from standard curve quantification of C_T-values were used to compare total expression levels of the target and candidate reference genes (Fig. 3.2) between the subpopulations PM, PA intertidal and PA subtidal. Values are related to total extracted RNA and given in arbitrary units (AU), and quantitative comparison of different genes is compromised by possible variations in reverse transcription efficiency of the mRNA transcripts.

β-Actin was very highly expressed in all measured samples (Fig. 3.2-left), with average values between 180 and 500 AU. Among untreated control animals, PM had a lower average actin expression than in the PA subpopulations, and over all treatments, PA animals from the intertidal showed significantly higher (one-way ANOVA $p<0.02$) *β-actin* expression values than subtidal and PM specimens.

Both inducible heat shock proteins *hsp70A* and *hsp70B* were strongly up-regulated during the experimental treatments (Fig. 3.2-middle). While in untreated control animals *hsp70A* was only present at background levels of 0.002 AU, *hsp70B* was expressed at 0.1-0.2 AU. Upon activation of the heat shock response, total expression levels of *hsp70B* were about 3-fold higher than *hsp70A* levels in PM and PA subtidal animals. PA intertidal limpets showed a significantly ($p<0.05$) weaker activation of *hsp70A*. Response was significantly stronger ($p<0.05$) in PM animals than in PA, with highest average levels at 20 AU (*hsp70A*) and 80 AU (*hsp70B*) as opposed to only 1 and 6 AU, respectively.
Heat shock cognate *hsc70* and Glucose-regulated protein *grp78* levels were comparatively stable at intermediate levels of 0.2-1.6 AU in all populations/treatment combinations.

Hypoxia-inducible transcription factor *HIF-1* and histone *H3* transcripts were present at a low level of 0.01-0.05 AU (Fig. 3.2-right). In untreated control groups, *H3a* expression was significantly higher ($p<0.05$) in PA subtidal animals than in the intertidal subpopulation.

3.2.3 Normalization to reference genes

For assessment of stability under the experimental treatment, expression values of the four candidate reference genes were checked in all samples (N=64) and similar recommendations were given by the two algorithms (GeNorm and NormFinder) (Tab. 3.2). Using values of all samples, GeNorm calculated *grp78* and *HIF-1* as the two most stable genes, and NormFinder ranked *grp78* as clearly the most stable and *HIF-1* as the second most stable gene. In both rankings, these were followed by *β-actin* and histone *H3*. NormFinder additionally proposed *grp78* and *H3* as a pair of genes which compensate for each other's deviation from the average.
When checking expression values for the three subpopulations separately, NormFinder ranked the single candidate reference genes in the same order, while GeNorm proposed *grp78* and *β-actin* for PM and *grp78* and *HIF-1* for both PA subpopulations. The same recommendations were given by NormFinder for a compensation pair of reference genes.

Based on these calculations and visual inspection of non-normalized expression data (cf. Fig. 3.2), we regard *grp78* as the most reliable reference and used it for normalization of moderately regulated genes. To improve statistical confidence, both *grp78* and *HIF-1* were used for normalization of the strongly up-regulated heat shock genes.

For comparison of untreated field levels of Hsp expression between the genes and subpopulations, stability of gene expression was checked among the control samples (N=12). GeNorm returned *grp78* and *β-actin* as most stable gene combination, followed by *HIF-1*, while NormFinder ranked genes in the order of *HIF-1*, *grp78* and *β-actin* and proposed *HIF-1* and *β-actin* as a pair with a considerably better stability value. Although rankings by the two programs differ in this case, the first three genes show similar stability. We

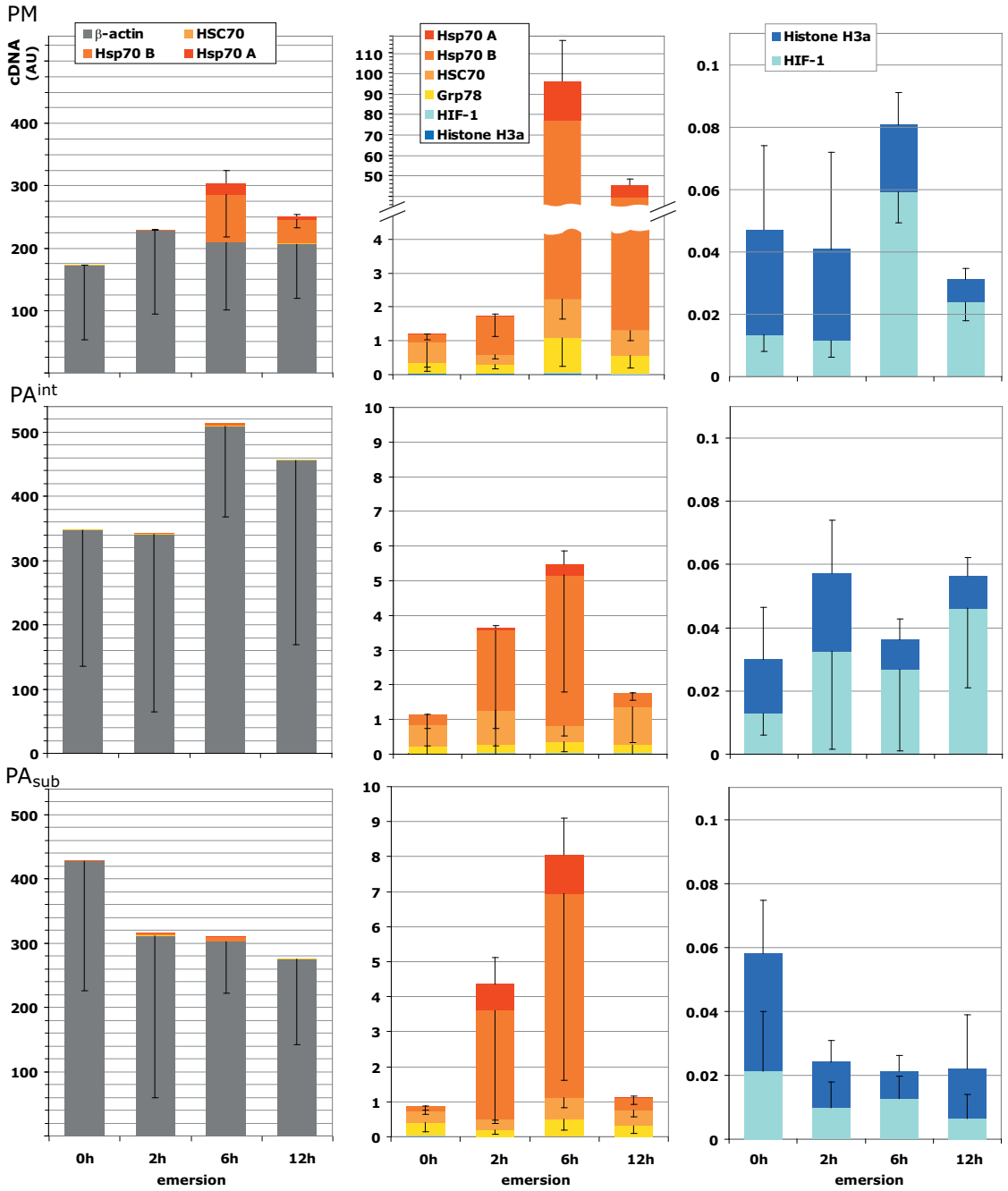

Figure 3.2: Quantification of total cDNA yields for targets of RT-qPCR: β-actin, *hsp70A*, *hsp70B*, *hsc70*, *grp78*, *HIF-1* and *histone H3*. Mean values for untreated control animals (0h) and after 2h, 6h and 12h air exposure treatments of the three populations Puerto Montt (PM), Punta Arenas intertidal (PA^{int}) and subtidal (PA_{sub}) are depicted on three different scales. Samples have been normalized to total RNA amount, standard deviations (biological variation) are symmetrical but only depicted in one direction, N=6 for each combination except N=4 for PA_{sub}-6h.

used two genes, *HIF-1* and *β-actin*, to achieve a sufficiently reliable comparison of *grp78* levels to the heat shock proteins.

Table 3.2: Stability ranking of candidate reference gene expression by two different algorithms. GeNorm calculates an average expression stability M based on the standard deviation between all genes and samples, while NormFinder returns a model-based stability value between subpopulation/treatment groups. Stability was calculated over all samples for normalization of experimental treatment effects and only for control animals for comparison of field levels. For control of possible variation between subpopulations, data for the three subpopulations Puerto Montt (PM), Punta Arenas intertidal (PA^{int}) and subtidal (PA_{sub}) were input separately (only first two ranks reported).

		GeNorm			NormFinder		
	N	rank	genes	M	rank	genes	stability
all	64	1st	grp78+HIF	1.1	1st	grp78	0.426
					2nd	HIF	0.562
		3rd	actin	1.34	3rd	actin	0.567
		4th	H3	1.53	4th	H3	0.759
					best pair	grp78+H3	0.412
controls	12	1st	grp78+actin	0.84	1st	HIF	0.448
					2nd	grp78	0.481
		3rd	HIF	1.16	3rd	actin	0.521
		4th	H3	1.51	4th	H3	0.781
					best pair	HIF+actin	0.306
PM	22	1st	grp78+actin	1.26	1st	grp78	0.380
					2nd	HIF	0.630
		3rd	HIF	1.41	best pair	grp78+actin	0.388
PA^{int}	20	1st	grp78+HIF	0.89	1st	grp78	0.297
					2nd	HIF	0.494
		3rd	H3	1.15	best pair	grp78+HIF	0.330
PA_{sub}	22	1st	grp78+HIF	0.86	1st	grp78	0.140
					2nd	HIF	0.290
		3rd	actin	1.06	best 2	grp78+HIF	0.163

3.2.4 Relative gene expression

Control levels of heat shock gene expression in field-acclimatized animals were calculated by standard curve quantification and normalized to *HIF-1* and *β-actin* (Fig. 3.3). No significant differences in control group Hsp levels between either the PM and PA locations or between the intertidal and subtidal subpopulation at PA could be found.

In most of the experimentally treated samples, a clear heat shock response was identified (Fig. 3.4), after normalization of relative expression values to the reference genes *grp78* and *HIF-1* using the REST 2009 program and testing for significance ($p<0.05$) with the integrated Bootstrap randomization test (Tab. 3.3). The response was characterized by

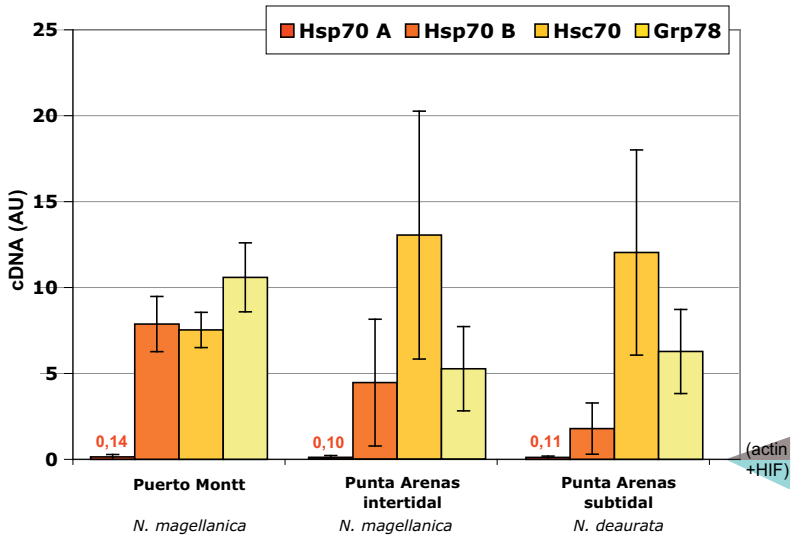

Figure 3.3: Heat shock gene expression in field-acclimatized *Nacella magellanica* and *N. deaurata* of three subpopulations. Normalized to reference genes *HIF-1/β-actin*. Bars show mean values of *hsp70B*, *hsc70* and *grp78* transcripts for untreated control animals (0h) and standard deviations (biological variation), N=4 for Puerto Montt, N=8 for both Punta Arenas subpopulations. Numbers shown are mean values for *hsp70A*.

up-regulations of inducible heat shock proteins by several orders of magnitude over control levels. While *hsp70A* and *hsp70B* regulation was apparently connected, the activation pattern of both genes differed markedly between subpopulations.
Both were found to be significantly up-regulated in all three air exposure treatments of the Puerto Montt population, reaching the highest average levels of all samples after 6h and 12h, 240–300-fold for *hsp70A* and 40–47-fold for *hsp70B*, with lower inter-individual variations than treatment groups from both PA subpopulations.
In subtidal PA animals, up-regulation levels of both genes were lower but still significant in all treatments, and we recorded the fastest onset of the heat shock response. Maximum expression levels of 87-fold (*hsp70A*) and 20-fold (*hsp70B*) occurred already in the 2h treatment, and *hsp70A* in this group as well as in the 12h treatment was significantly higher than in intertidal PA specimens. Intertidal animals from PA displayed a significant up-regulation of *hsp70A* in the 2h treatment, followed by a moderate further increase of both *hsp70A* (16-fold) and *hsp70B*(9-fold) after 6h.

All significant differences in expression of *hsp70A* and *hsp70B* were also observed at comparable levels in REST without normalization. Similar results were obtained using standard curve quantification (Fig. 3.5), but generally at higher up-regulation levels and with higher inter-sample variations, therefore only REST values are described here. Testing values calculated via standard curves with two-way ANOVA between subpopulations and treatments, only the 6h ($p<0.001$) and 12h ($p<0.01$) response of both Hsps at PM and 6h up-regulation of *hsp70B* at PA_{sub} ($p<0.05$) were statistically significant.

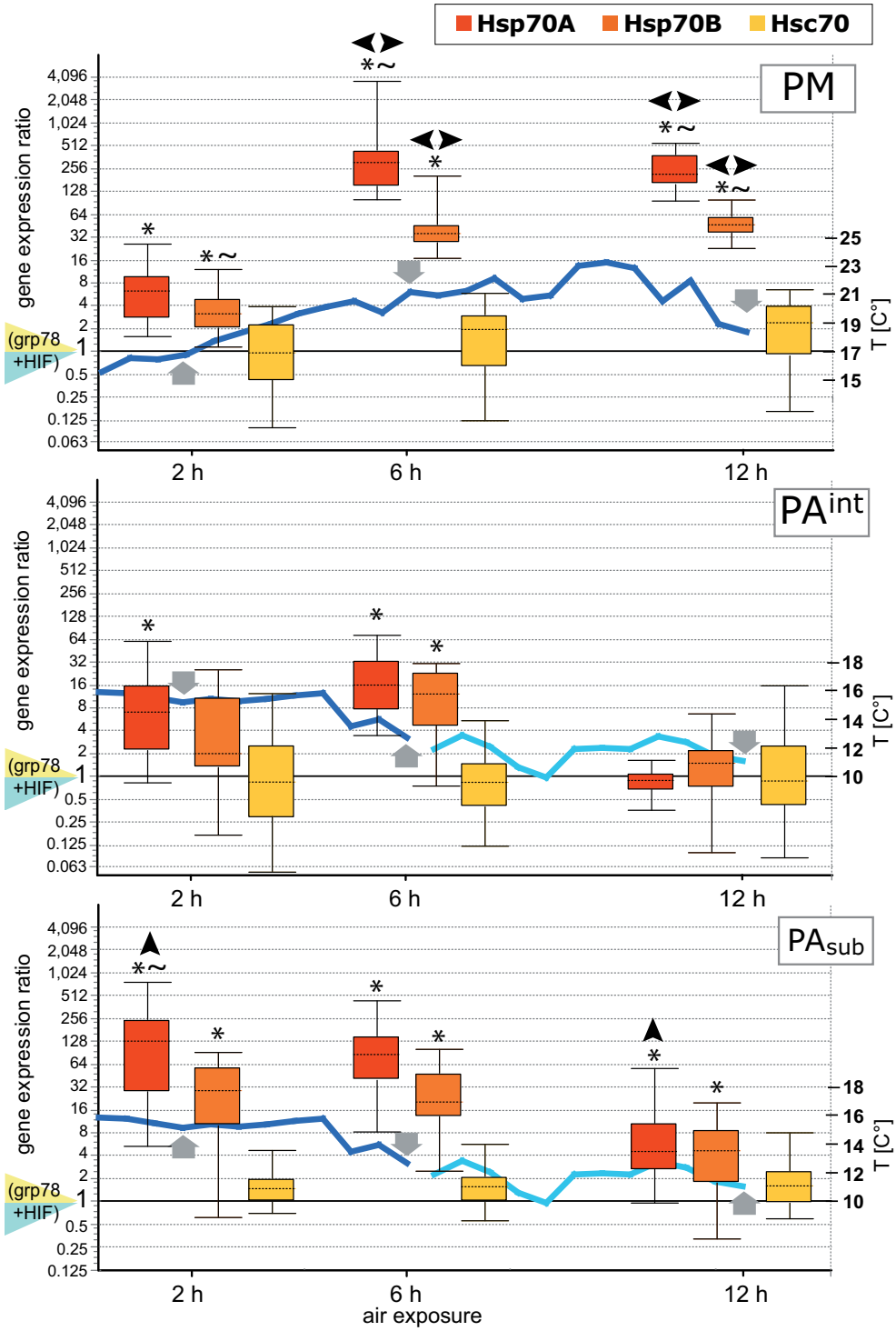

Figure 3.4: Induction of the Hsp70 stress response under treatments of 2h, 6h and 12h air exposure, compared to air temperature (T). *Hsp70A, hsp70B* and *hsc70* expression (normalized to reference genes *grp78/HIF-1*) relative to untreated control animals (left scale, logarithmic), calculated using REST 2009, for the three populations Puerto Montt (PM), Punta Arenas intertidal (PA^{int}) and subtidal (PA_{sub}). Coloured boxes represent 50% of values with median value (dotted line), whiskers are extreme values, N=6 for each group except N=4 for 6h-PA_{sub}. Marked values are significantly different ($p<0.05$) to untreated control animals (*), to water control animals (∼), among intertidal position PA_{sub}–PA^{int} (vertical triangle), and between locations PM-PA (horizontal triangles). Lines represent air temperatures in the course of the experiment (right scale, linear), arrows mark experimental time points.

Table 3.3: Confidence intervals (C.I.) and statistical significance of heat shock gene up-regulation under treatments of 2h, 6h and 12h emersion (normalized to reference genes *grp78* and *HIF-1*). Average gene expression (Expr.) for each subpopulation Puerto Montt (PM), Punta Arenas intertidal (PA^{int}) and subtidal (PA_{sub}), relative to untreated control animals. N=6 for each treatment/population combination except N=4 for 6h-PA_{sub}. Values in bold type are significantly up-regulated (P(H1)<0.05), as indicated by random allocation between groups (2000 Bootstrap iterations). All values calculated using REST 2009 (RG Mode).

	2h			**6h**			**12h**		
	Expr.	95% C.I.	P(H1)	Expr.	95% C.I.	P(H1)	Expr.	95% C.I.	P(H1)
PM									
hsp70A	**5.38**	1.89 – 22.01	**<0.001**	**314.09**	107.45 – 2,926.19	**0.008**	**240.43**	110.77 – 557.52	**<0.001**
hsp70B	**3.25**	1.55 – 10.94	**<0.001**	**40.53**	18.17 – 189.07	**0.008**	**47.16**	24.14 – 93.84	**<0.001**
hsc70	0.87	0.08 – 4.95	0.831	1.37	0.18 –5.07	0.559	1.62	0.19 –6.26	0.396
PA^{int}									
hsp70A	**6.45**	0.93 – 53.19	**0.025**	**15.7**	3.72 –66.35	**0.030**	0.84	0.4 –1.53	0.425
hsp70B	2.79	0.17 – 23.26	0.189	**8.7**	1.05 –29.36	**0.042**	1.13	0.13 –6.32	0.820
hsc70	0.94	0.06 – 11.98	0.957	0.79	0.14 –4.73	0.706	0.99	0.1 –12.51	0.995
PA_{sub}									
hsp70A	**86.95**	7.4 – 638.26	**0.004**	**75.2**	11.86 – 395.97	**<0,001**	**5.46**	1.26 –40.74	**0.018**
hsp70B	**18.77**	1.78 – 82.81	**0.014**	**21.78**	2.79 –96.28	**<0,001**	**3.66**	0.36 –17.81	**0.047**
hsc70	1.44	0.7 –4.32	0.211	1.53	0.6 –5.22	0.232	1.61	0.61 –6.14	0.203

For the analysis of more subtle expression changes in the genes *hsc70*, *HIF-1*, *β-actin* and *H3* during air exposure, only the most stable gene *grp78* was used as a reference gene (cf. 3.1.2). Normalized and non-normalized mean regulation levels obtained for these genes were similar between the two applied methods, REST 2009 (Fig. 3.6) and standard curve quantification (results not shown). Non-normalized expression data show a significant up-regulation of *HIF-1* ($p<0.05$ REST) in 6h treatments from PM and in 12h-treated intertidal specimens from PA, but these changes lose their statistical significance upon normalization to *grp78* (Fig. 3.6).

In histone *H3* levels, down-regulation under the experimental treatment was observed in the PM 12h treatment at about 30% of the untreated control expression. In subtidal PA animals of 6h and 12h treatment durations, mean values of *β-actin* expression were reduced to around 50-70%. This reduction was significant compared to water covered control animals (REST $p<0.025$) but not against untreated control animals (Fig. 3.6).

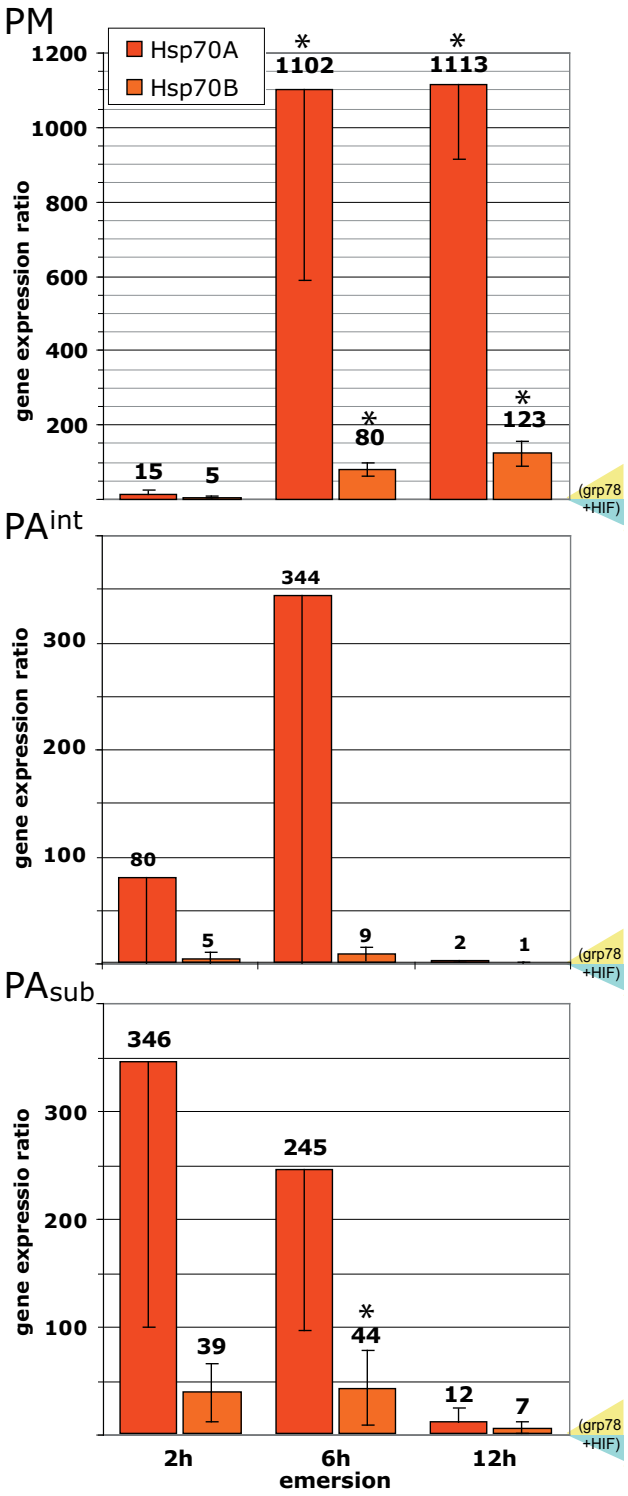

Figure 3.5: Up-regulation of *hsp70A* and *hsp70B* expression under treatments of 2h, 6h and 12h air exposure, as calculated by standard curve quantification. Normalized to reference genes *grp78/HIF-1*). Mean values of relative expressions compared to untreated control animals for the three subpopulations Puerto Montt (PM), Punta Arenas intertidal (PA^{int}) and subtidal (PA_{sub}). N=6 for each group except N=4 for 6h- PA_{sub}. Standard deviations (biological variation) are symmetrical but only depicted in one direction for *hsp70A*. * Marked values are significantly different to untreated control animals ($p<0.05$, two-way ANOVA)

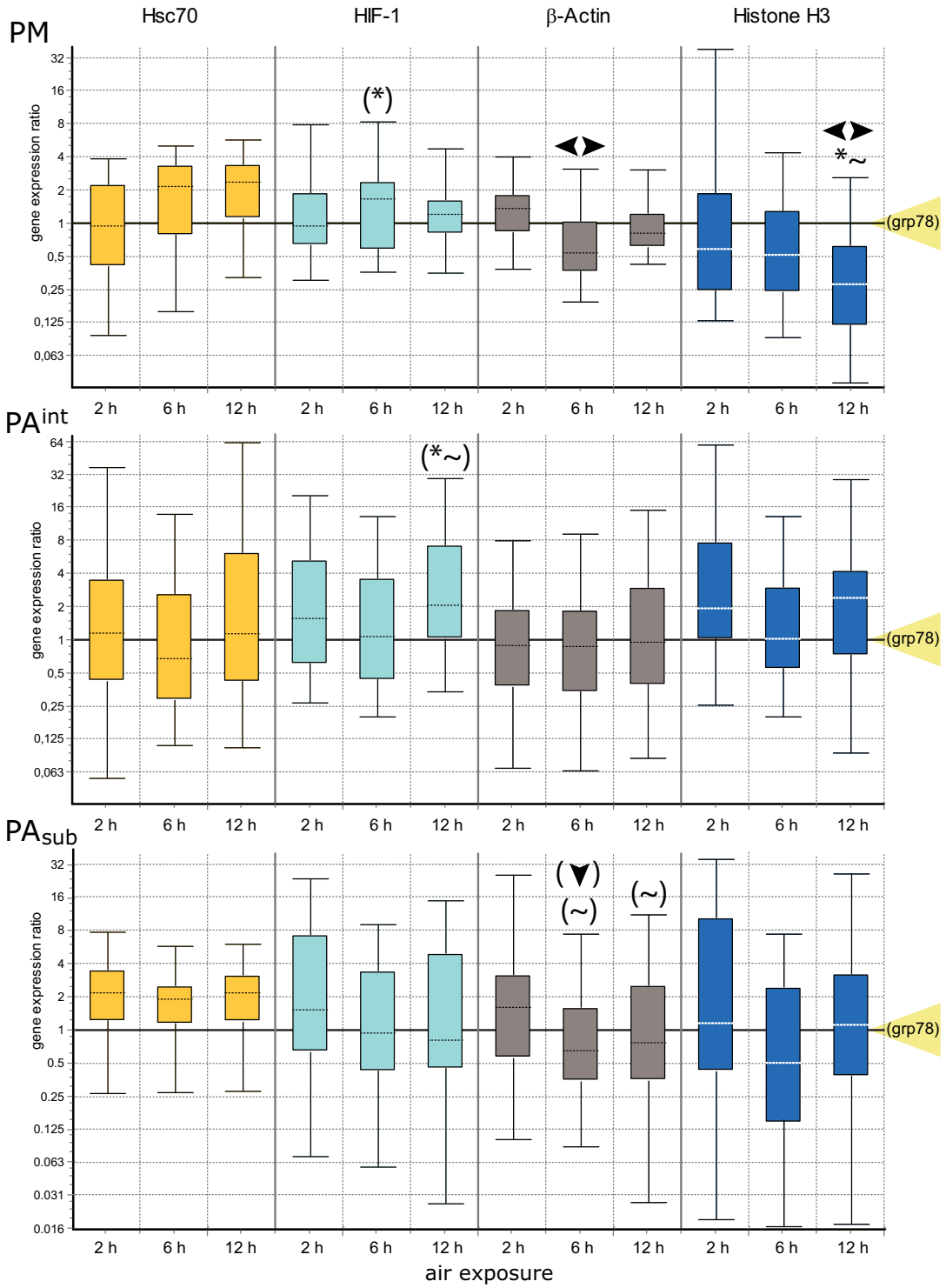

Figure 3.6: Change of *hsc70*, *HIF-1*, *β-actin* and *histone H3a* expression under treatments of 2h, 6h and 12h air exposure. Normalized to reference gene *grp78*. Relative expressions compared to untreated control animals, calculated using REST 2009, for the three populations Puerto Montt (PM), Punta Arenas intertidal (PA^{int}) and subtidal (PA_{sub}). Coloured boxes represent 50% of values with median value (dotted line), whiskers are extreme values, N=6 for each group except N=4 for 6h- PA_{low}. Marked values are significantly different (Bootstrap randomizationp $p<0.05$) to untreated control animals (*), water control animals (∼), intertidal position PA_{sub}–PA^{int} (vertical triangle), location PM-PA (horizontal triangles). Symbols in brackets indicate significant differences in non-normalized data.

3.3 Allelic diversity of Hsp70 genes

Genomic *hsp70A* and *hsp70B* sequences from the two experimental sites and six additional locations revealed allelic differentiation among the Patagonian *Nacella* samples.
In the 693 bp alignment of *hsp70A* gene sequences from 182 specimens, 27 polymorphic sites were identified. 15 of these follow an unambiguous distribution pattern, with five leading to amino acid differences in the translated protein. Samples were assigned to two allele groups A1 and A2 according to these 15 sites, based on the assumptions that these fragments represent distinct lineages and no recombination is present. 44 samples were designated as heterozygote A1A2, because all 15 sites were marked as heterozygous for the two alternative bases (Appendix 4.2). These two split pseudoallele groups were used for further analyses of F_{st} estimation. In the haplotype network, the two assigned allele groups are separated at a connection limit of 95% parsimony, and further differentiation is shown according to the other 12 polymorphisms in the *hsp70A* alleles (Fig. 3.7).

The 629 bp alignment of *hsp70B* from 64 specimens showed 12 polymorphic sites. Five of these displayed an unambiguous pattern, of which two were non-synonymous. I assigned the samples into two preliminary groups B1 and B2 and tested the *hsp70A* and *hsp70B* allele groups for linkage disequilibrium among the 33 samples where both isoforms have been sequenced. The null hypothesis that loci are independent could not be rejected, with P=0.10 over all samples and P=0.10 in the *N. deaurata* subpopulation at PA (N=13). No further analyses on the *hsp70B* alleles were performed because of their low differentiation and low confidence in assignment of the allele groups.

The two *hsp70A* allele groups dominate the Pacific *N. magellanica* and Atlantic *N. deaurata* populations, respectively (Tab. 3.4). While all sampled *N. magellanica* individuals from the outer Pacific coast (PM, US, SO) are homozygous for the A1 allele, the easternmost *N. deaurata* population on the Falkland Islands is dominated by the A2 allele, with one third A1A2 heterozygotes. Between these two extremes, in the Strait of Magellan populations, distribution changes progressively from A1 to A2 alleles (Fig. 3.8).

Overall estimation of population differentiation according to the F_{st} estimate theta (Weir and Cockerham 1984) was $\Theta = 0.563$ (Std. error 0.094). The score tests for Hardy-Weinberg equilibrium detected no significant deviations within the sampled subpopulations (cf. tab. 3.4).

Estimates of allelic divergence for the *hsp70A* locus are based on the two allele groups and 12 polymorphic sites in the sequence alignment using a distance matrix method. Pairwise F_{st} estimates evidence a clear separation between the *N. magellanica* and the *N. deaurata* subpopulations in all locations, ranging from 0.36 between the two species in the Western and Central Strait of Magellan to 0.86 between the two most geographically separated populations in Puerto Montt and on the Falklands. While no significant differentiation

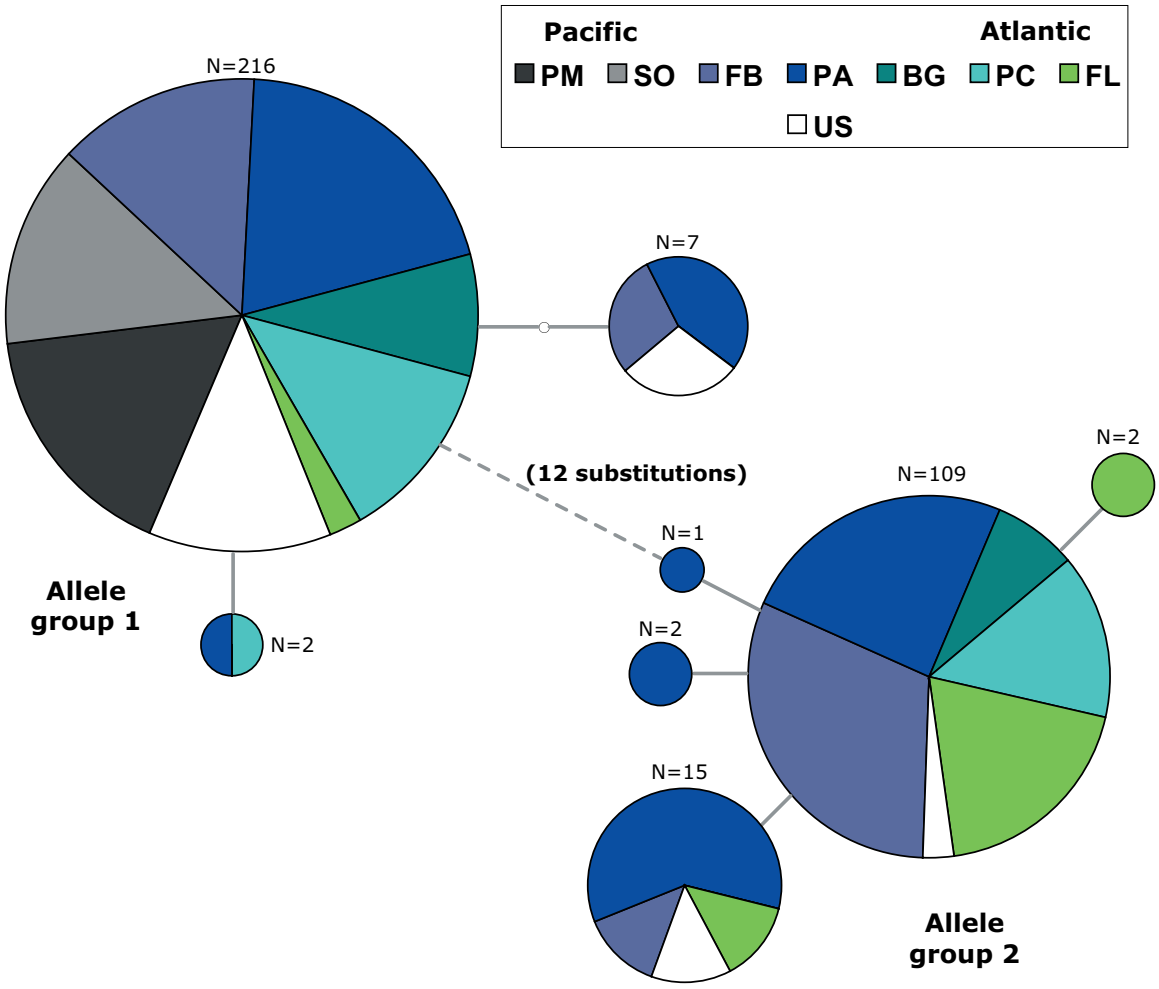

Figure 3.7: Distribution of *hsp70A* haplotypes in Magellanic *Nacella*. Statistical parsimony network at 95% connection limit, based on split allele groups and polymorphisms within groups. Haplotypes are divided by sampling sites, N is given of sampled individuals of both *N. magellanica* and *N. deaurata*.

among the sampled *N. deaurata* populations was detected, a major distribution shift in *N. magellanica* occurred in the Strait of Magellan between Punta Arenas and Bahia Gregorio.

I used additional standardized pairwise F'_{st} estimates from eight microsatellites (Poehlmann and Held, in prep.) for comparison, assuming them to represent a neutral baseline of genetic differentiation. In total, these show markedly higher allelic identity than the two *hsp70A* alleles and divergence follows geographical distance. Marked differences are present in the F_{st} estimates of both markers between intertidal *N. magellanica* and subtidal *N. deaurata* in the Strait of Magellan, i.e. in the experimental population at Punta

Arenas and in Fuerte Bulnes (Fig. 3.9). Furthermore, the Central-Eastern *N. magellanica* populations of Bahia Gregorio and Punta Catalina show disparities in comparison to the sites from the Pacific West coast.

Table 3.4: Distribution of *hsp70A* and *hsp70B* allele groups in *Nacella* samples from Patagonia. Sample numbers for homozygotes and heterozygotes of two allele groups, one predominant in *N. magellanica* (*N.ma*) from the Pacific coast (A1 and B1), and one predominant in *N. deaurata* (*N.de*) from the Atlantic coast (A2 and B2). Locations are sorted from west to east. Heterozygote deficit F_{is} is estimated for *hsp70A* in polymorphic subpopulations with 13 specimens or more. Homozygote and heterozygote assignment is based on ambiguous peaks in sequence chromatograms, for 15 sites showing an unequivocal pattern in *hsp70A*, and four sites in *hsp70B*.

Hsp70A		Puerto Montt	Seno Otway	Ushuaia	Fuerte Bulnes	Punta Arenas	Bahia Gregorio	Punta Catalina	Falkland Islands
N.ma	A1A1	18	15	13	12	18	5	7	
	A1A2	0	0	0	0	3	8	14	
	A2A2	0	0	0	0	0	0	1	
	$\boldsymbol{F_{is}}$					-0.05 (P=0.93)	-0.4118 (P=0.21)	-0.3548 (P=0.11)	
N.de	A1A1			1	1	1			0
	A1A2			1	6	7			5
	A2A2			4	15	17			10
	$\boldsymbol{F_{is}}$			0.6154 (P=0.27)	0.1064 (P=0.54)	0.0718 (P=0.58)			-0.1667 (P=0.67)
Hsp70B									
N.ma	B1B1	7	7	6	1	6		8	
	B1B2	0	0	0	0	1		0	
	B2B2	0	0	0	0	0		0	
N.de	B1B1				0	0			0
	B1B2				6	7			3
	B2B2				0	7			3

For phylogenetic comparison of the three cytosolic *Nacella* Hsp70 isoforms and confirmation of their paralogy, a longer sequence alignment was analysed. The 960 bp sequences were derived by extension of the 693 bp consensus sequences of the two assigned *hsp70A* and *hsp70B* allele groups, by sequencing of two typical fragments with a reverse primer. As an outgroup, two intertidal and two subtidal samples from *N. concinna* were used, which contained no polymorphisms. For *hsc70*, four samples from *N. magellanica* (PM), *N. deaurata* (PA) and *N. concinna* were sequenced.

The Hsp70 sequences exhibit an overall similarity of 61%. One 3 bp insertion in *hsp70B* was detected, and the three Hsps showed very high interspecific conservation: *hsp70B* and *hsc70* share 98% and 99% sequence identity, respectively, while *hsp70A* is more variable with 96.5% conservation between the three Nacellid species.

The Maximum parsimony tree of the heat shock nucleotide sequences supports the paral-

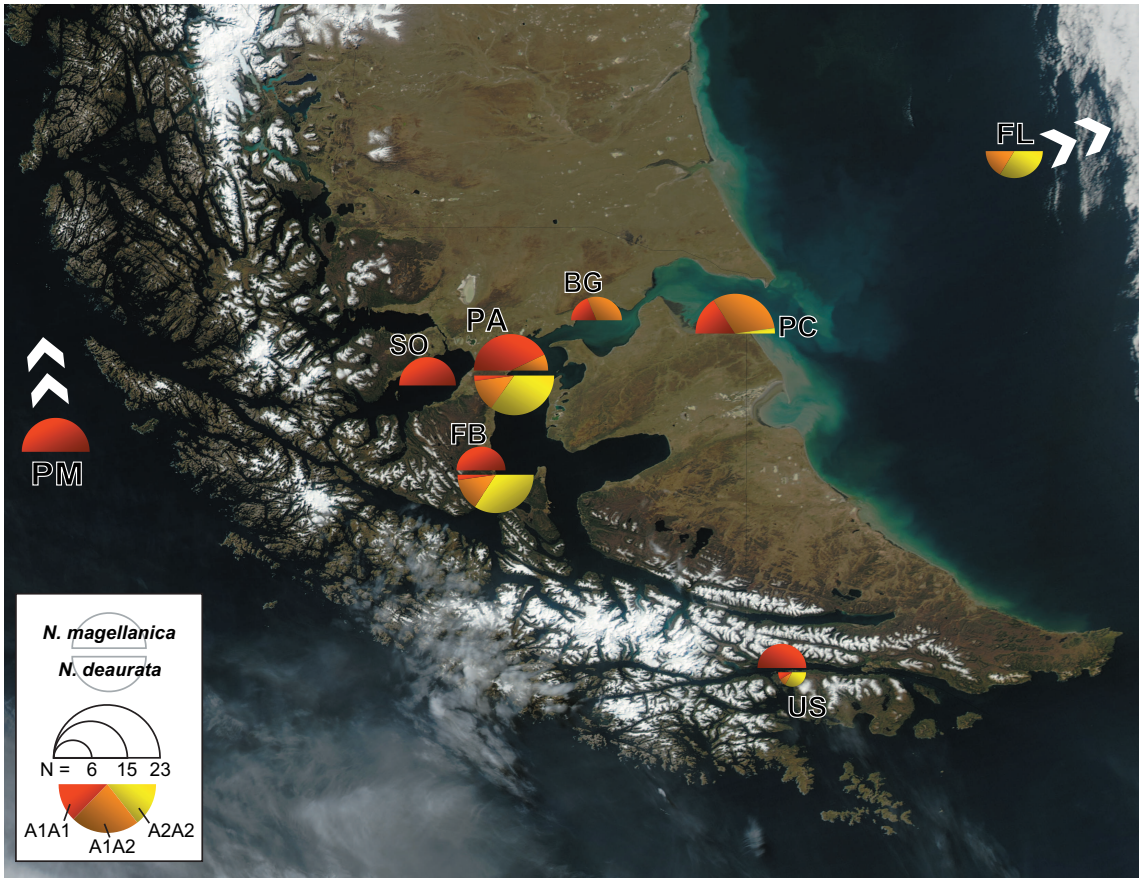

Figure 3.8: Distribution of *hsp70A* allele groups A1 and A2 in *Nacella* in the Magellan Province. Satellite image (NASA/MODIS) with sampling locations. Half circles give percentage of homozygotes A1A1 (red), heterozygotes A1A2 (orange) and homozygotes A2A2 (yellow) in the sampled subpopulations. Upper half circles give distribution for intertidal *N. magellanica*, lower half circles for subtidal *N. deaurata*. Sizes of half circles correspond to sample sizes N=6–23, for exact numbers see fig. 3.4, for names and coordinates of locations see 2.1

ogy of *hsp70A*, *hsp70B* and *hsc70,* and groups together the inducible genes *hsp70A* and *hsp70B* (Fig. 3.10). While in *hsp70B* and *hsc70*, a larger genetic distance of *N. concinna* to the Patagonian subspecies is clearly indicated, a lower distance of *hsp70A* from *N. magellanica* to the Antarctic congener than to its Magellanic relative is calculated but only supported by a Bootstrap value of 76. Sequences of all three genes unambiguously contained the primer regions used for qPCR, confirming specificity of amplification in the assessment of gene expression levels.

The translated sequences of 320 amino acids represent 40-50% of the protein, as estimated by alignment with complete coding sequences of mollusc Hsp70s available in GenBank, and form part of the ATP-binding region. The Hsp70A fragment contains seven variable sites, while Hsp70B has two and Hsc70 shows no amino acid substitutions (Tab. 3.6).

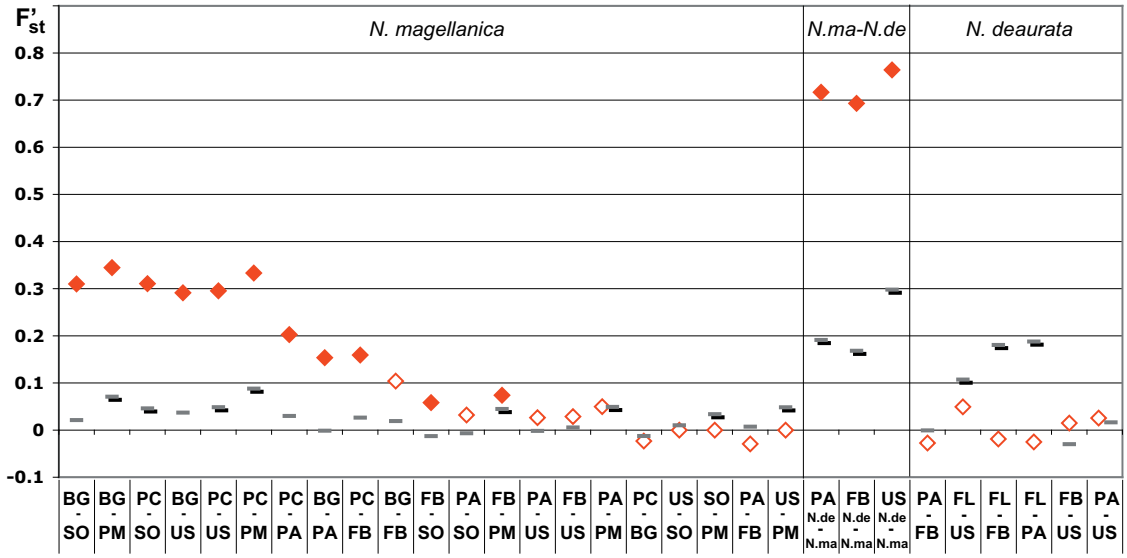

Figure 3.9: Comparison of divergence in the *hsp70A* locus and a neutral baseline of genetic differentiation in Patagonian *Nacella*. Pairwise F_{st} estimates for *hsp70A* allele groups (diamonds) and standardized pairwise F'_{st} estimates for additional microsatellite markers (lines) in *Nacella magellanica* (*N.ma*) and *N. deaurata* (*N.de*) subpopulations. Depicted are values among populations of *N.ma*, between *N.ma* and *N.de* at the same location, and between *N.de* populations, sorted according to difference in F_{st} estimates within groups. Solid diamonds and double lines show significant divergence between subpopulations, see 2.1 for site names and coordinates.

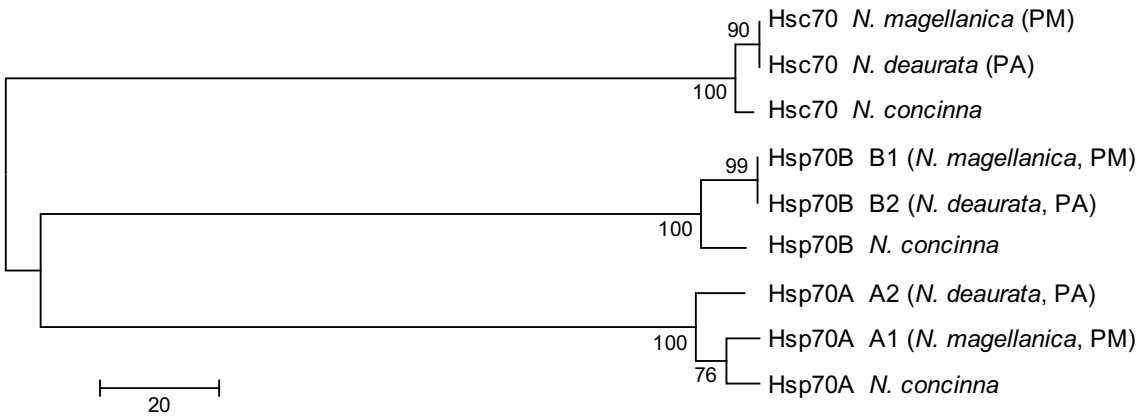

Figure 3.10: Paralogy of the Hsp70 genes in Patagonian *Nacella*. Phylogenetic tree (Maximum Parsimony, mid-point rooted) of a 960 bp mRNA sequence fragment of the heat shock genes *hsp70A*, *hsp70B* and *hsc70* in *Nacella* limpets from Northern Patagonia (PM, Puerto Montt), Strait of Magellan (PA, Punta Arenas) and Antarctic Peninsula (*N.concinna*, King George Island). Support values for nodes were calculated by 1000 Bootstrap iterations.

Table 3.5: Allelic differentiation of *hsp70A* in *Nacella* from the Magellan Province. Pairwise F_{st} estimates for *hsp70A* allele frequencies, based on assignment to two allele groups and a sequence distance matrix between sampled subpopulations of *Nacella magellanica* (*N.ma*) and *N. deaurata* (*N.de*). Values in bold type are significant ($p<0.05$). See 2.1 for site names and coordinates.

	PM *N.ma*	SO *N.ma*	US *N.ma*	FB *N.ma*	PA *N.ma*	BG *N.ma*	PC *N.ma*	US *N.de*	FB *N.de*	PA *N.de*
SO *N.ma*	0.0000									
US *N.ma*	0.0000	0.0000								
FB *N.ma*	**0.0740**	**0.0584**	0.0287							
PA *N.ma*	0.0498	0.0321	0.0263	-0.0293						
BG *N.ma*	**0.3447**	**0.3101**	**0.2912**	0.1040	**0.1536**					
PC *N.ma*	**0.3332**	**0.3106**	**0.2953**	**0.1592**	**0.2026**	-0.0232				
US *N.de*	**0.8196**	**0.7939**	**0.7641**	**0.5687**	**0.6363**	**0.1646**	0.1341			
FB *N.de*	**0.8093**	**0.7953**	**0.7797**	**0.6931**	**0.7261**	**0.4174**	**0.3588**	0.0149		
PA *N.de*	**0.7949**	**0.7805**	**0.7646**	**0.6825**	**0.7167**	**0.4151**	**0.3629**	0.0256	-0.0274	
FL *N.de*	**0.8567**	**0.8434**	**0.8292**	**0.7318**	**0.7624**	**0.4472**	**0.3805**	0.0493	-0.0189	-0.0251

Table 3.6: Amino acid differences in the partial *hsp70A* and *hsp70B* sequences from three *Nacella* species. Depicted are non-synonymous substitutions from translated mRNA sequences, the 320aa-alignments putatively start approx. 70 amino acids from the N-terminus and form part of the ATP-binding domain. Bold type marks residues with different chemical properties.

	hsp70A							***hsp70B***	
position	35	38	55	167	170	206	238	86	214
group 1 *(N. magellanica)*	Asp	**Ala**	Asp	Thr	Asp	**His**	**Lys**	**Ile**	**Ser**
group 2 *(N.deaurata)*	**Asn**	Thr	Glu	Ser	Glu	Tyr	Asn	Thr	Ala
N.concinna, Antarctica	Asp	Thr	Asp	Thr	Asp	Tyr	Asn	Thr	Ala

4 Discussion

4.1 Methodological issues and implications for the quantification of the heat shock response

The results of my study illustrate that the choice of an adequate reference gene for the quantification of gene expression by real-time RT-qPCR is not trivial. In biomedical research, quantification and evaluation of several reference genes is regarded as an essential part of the assay, to provide a reliable baseline for normalization of the qPCR data (Ginzinger 2002, Huggett et al. 2005, Bustin et al. 2009). In contrast, in ecophysiological RT-qPCR studies of animal stress responses, unsubstantiated reference to a single classical 'housekeeping' gene is still common (Clark et al. 2008b, Fangue et al. 2006). In studies of non-model organisms, higher effort is required to design primers, thus the number of quantified genes is typically lower. When limited expression data for comparison is available, reliability of results can be put at risk by normalization to genes that are generally regarded as stably expressed, but might be strongly influenced by stressful experimental conditions. Furthermore, the individual variation inherent in natural samples will usually be markedly higher than in studies of cell cultures, due to genetic and habitat heterogeneity. If gene expression is to be compared between populations under different ecological conditions or between species, the task is not only to find a stable gene against the background of phenotypic plasticity, but also against genetic variation. Thus, a balance between reliability of the data and economical considerations has to be reached.

I quantified a total of seven genes and compared two popular mathematical approaches for assessment of expression stability, GeNorm and NormFinder. When the tested gene and sample numbers are relatively low, the danger of corrupting the statistics of the available ranking programs by choosing genes that are actually co-regulated is higher. The two algorithms are based on different assumptions, and applying physiological reasoning and creating an initial set of reference candidates is necessary to achieve reliable results. The GeNorm algorithm calculates the relative expression stability between candidate genes, and deviating genes are consecutively excluded, assuming that when genes show a very similar expression profile across all samples, they are stable (Vandesompele et al. 2002). In my set of quantified genes, four out of seven genes code for molecular chaperones. This similarity in physiological function increases the chance of co-regulation, making it highly

likely that the algorithm would detect two of these as being stable. Using the values of all genes, GeNorm identified *hsc70* and *HIF-1* as the most stable genes (results not shown), but these two genes might also be co-regulated (Fig. 3.6). Furthermore, because my data set consists of three *Nacella* subpopulations that potentially have a different genetic setup, I had to double-check stability of the reference genes for each subpopulation separately. The NormFinder program offers the feature of comparison between groups, making its use more convenient when comparing different populations or even species. It calculates a theoretical baseline by comparing all regulations, thus it assumes an average of all genes to be stable (Andersen et al. 2004). Inclusion of the heat shock protein genes, which show extremely strong up-regulation, would lead to a substantial upward-shift of the average baseline, and NormFinder proposes *hsc70* as the most stable gene, and *hsp70B* and *β-actin* as best combination. From non-normalized data, I suspect *hsc70* to be slightly up-regulated under the experimental treatments, and in my opinion, using the obviously extremely variable *hsp70B* for normalization would jeopardize the other genes' expression values.
When restricting the input to only one chaperone (*grp78*) and the three other genes, both programs produced very similar results (Tab. 3.2). I used *grp78* and *HIF-1* for a common basis of heat shock response levels in all animals. For the PM population, where *grp78* and *β-actin* were ranked as the most stable genes when checking the values in separate groups, I verified that all conclusions would also hold with this reference combination. For analysis of the more subtle changes in the non-HSR genes, only the most stable gene *grp78* was used as a reference. Comparing these choices with the results of using other reference gene combinations, I confirmed that this was also the normalization strategy that produced the lowest standard deviations in all treatment averages.
In conclusion, to achieve a reliable normalization with a limited number of genes, candidate reference genes should be defined *a priori* on the basis of physiological considerations, and represent a well-balanced set of genes from different functional groups, as far as this is possible and known beforehand. Especially in habitats with periodically changing conditions like the intertidal, temporal splitting of basic physiological functions and thus differential regulation of genes can be expected (Gracey et al. 2008). Genes for which regulation is suspected should be defined as target genes and *a priori* excluded from the stability calculations, as the established algorithms are sensitive to errors introduced by co-regulated genes (GeNorm) and genes with extremely high regulation ratios (NormFinder), respectively. In addition, results have to be compared between separated subpopulations of the samples to account for possible genetic divergence.

It may be helpful to abandon altogether the idea of a universally stable 'housekeeper' gene - rather, it is a question of finding a reference that (1) is sufficiently stable under the experimental conditions and (2) makes physiological sense in the light of the question investigated and the statement to be made. *grp78* and *HIF-1* should provide a reliable

measure of the induced stress response levels. Hypoxia Inducible Factor 1 subunit α (*HIF-1*) is a central transcription factor for responses to low oxygen levels, and is reported to be constitutively transcribed and, instead, regulated only on the protein level (Pugh and Ratcliffe 2003, Wenger 2002). The second reference gene *grp78* is a constitutive molecular chaperone of the Hsp70 class located in the endoplasmatic reticulum and involved in protein secretion (Haas 1994). It is reported to be relatively stable under short-term stress in *Nacella* (Clark et al. 2008a). It is impossible to definitely exclude that these reference genes are up- or down-regulated under the experimental conditions. Nevertheless, they do not show unambiguous changes or correlations in non-normalized expression levels (Fig. 3.2). Because a highly reproducible automated homogenisation method was employed, and tissue samples as well as the extracted RNA were protected against degradation during the entire process, I regard this as strong indication for stability of the reference genes. Stability rankings and physiological results (Fig. 3.6) show that β-actin is a questionable reference gene in quantification of the heat shock response. In our data, some mean up-regulation levels and all standard deviations of treatments increase drastically when using *β-actin* as a reference (Appendix 4.1).

A general methodological problem for quantification of expression changes under stress conditions arises from possible effects on overall transcription levels and/or rRNA/mRNA ratio. In the RT-qPCR assay, it is a usual step to normalize to total RNA or mRNA amounts, to account for differential yields of the extraction procedure and ensure reproducible cDNA synthesis. Therefore, all gene expression levels in my study, even when not normalized to a reference gene, have to be understood as relative to total RNA, and overall transcription levels or mRNA/rRNA ratio cannot be quantified. Since the synthesis of heat shock proteins in high amounts can have a strong effect on the overall protein synthesis of a cell by using up a substantial portion of the cellular energy or by cytotoxic effects (Krebs and Feder 1997a, Feder and Hofmann 1999), normalization may lead to overestimation of Hsp expression levels, and also upscale biological and pre-measurement technical variation. I suspect this to be partly responsible for the high individual variations that are observed in my and in many other studies (Clark and Peck 2009b). This problem can also be mitigated by choosing appropriate reference genes that are not strongly affected by down-regulation. The different approaches of the two normalization programs mirror the basic question, if gene expression can be described as an absolute value or if it makes more sense to refer to regulation as relative to a certain average level of transcription.

Two different approaches for extraction of quantities from the raw fluorescence curve have been applied in this study, which both have their own advantages. The standard curve method allows to judge quality of the primers and reliability of the results over the quantification range. Nevertheless, actual reaction efficiencies might deviate from the standard values. The Second Derivative Maximum method (Luu-The et al. 2005) as implemented in the RotorGene software addresses this issue and calculates an individudal reaction ef-

ficiency for each sample. The REST 2009 program uses the CP values from the SDM method and applies posterior Bootstrap replications to test the data for significant regulations without assuming normal distribution. More statistically significant expression changes could be detected by this approach, and I suspect the SDM method to be partly accountable for the lower variation within treatments in the calculated data. Unfortunately, the REST software returns only up-regulation ratios relative to a control group, while total expression values calculated by the standard curve method have been useful by providing a basis for stability calculations, and for comparison of Hsp70 expression levels under natural acclimatization.

In the 12h treatment of both PA subpopulations, transcription of inducible Hsps was back to sub-maximal levels. This might indicate that the heat shock response had already passed its peak level before 12h of emersion, an effect that is not visible in PM animals, where a stable plateau of Hsp expression is maintained after 12h. This observation might be influenced by differences in the experimental protocol: 12h treatments at PA had to be conducted on another day, when air temperatures did not reach the same levels as in the 2h and 6h treatments (Fig. 2.3). Thus, the reduced values in the PA 12h treatments are possibly due to the temperature-dependent nature of the stress response and not to an early end of induction after prolonged emersion. Nevertheless, comparison of both 12h groups in the experiment at PA shows that *hsp70A* and *hsp70B* levels of the subtidal animals are significantly increased, while the intertidal group is at control levels.

4.2 The heat shock response in Patagonian *Nacella*

Massive responses of both inducible heat shock genes *hsp70A* and *hsp70B* were detected in the southern South American limpets under a near-natural experimental setup and after performing careful normalization and quantification. Induced expression levels in the subpolar Punta Arenas population were similar to those documented as a response to tidal emersion for the close relative *N. concinna* from Antarctica (Clark et al. 2008a), and induction in the more Northern Puerto Montt population was even higher. It is not always the case that such levels can be triggered by realistic conditions: the threshold for induction in laboratory studies is often a sudden temperature increase of at least 8-15°C, which represents thermal changes only seldomly or never experienced by marine invertebrates under their natural environmental conditions (Clark et al. 2008a, Dong et al. 2008, Hamdoun et al. 2003). This might indicate that the heat shock response is not of actual ecological significance in these cases, or that the triggers for induction are more complex and were not adequately modelled by the laboratory conditions. On the other hand, laboratory acclimation to a constant temperature regime prior to experiments can result in lower constitutive Hsp levels compared to the variable intertidal environment, leading to

an overestimation of the stress induced up-regulation levels (Tomanek and Somero 1999). Hence, it is often difficult to connect results from laboratory experiments to the natural role of Hsps in situ. The control animals were naturally acclimatized to the tidal rhythm, and emersion experiments were conducted on site with freshly collected individuals, minimizing potential influence of transport or acclimation. Care has been taken in the choice of reference genes not to overestimate up-regulation levels. For statistical confidence, I used the two most stable genes in the assay as a reference to quantify the heat shock proteins. One of those, *grp78*, is a constitutive chaperone of the Hsp70 class, which should provide a conservative measure of stress response (cf. 4.1).

As a general caveat, trends in mRNA transcript levels may not be fully indicative of protein levels or activity. Although mRNA can be a highly sensitive indicator of physiological changes, many other levels of regulation are known (Gracey and Cossins 2003, Feder and Walser 2005). But studies suggest that in the heat shock response, Hsp expression is activated rapidly (Tomanek and Somero 2000) and negatively auto-regulated when Hsp levels reach a certain limit, indicating that mRNA levels are tightly regulated (Feder and Hofmann 1999). For the measurement of constitutive expression levels, control limpets were sampled just before tidal emersion and, to my knowledge, so far no endogenous tidal rhythm in Hsp synthesis has been found in other intertidal organisms (Todgham et al. 2006, Hofmann and Buckley 2002).

The different Hsp70 genes are interconnected through a common regulatory basis, HSF1, and their combination has been shown to be involved in phenotypic plasticity of the stress response (Tomanek 2010). While the constitutive chaperones *hsc70* and *grp78* were on average evenly expressed in the treatment groups, strong up-regulations were recorded in the inducible heat shock proteins *hsp70A* and *hsp70B*. These differences in the expression patterns give indications about the physiological role of the individual proteins and their potential for adaptive evolution. Partial sequencing of the three cytosolic Hsp70 isoforms confirmed their high conservation and close relationship to the orthologous genes originally described in *Nacella concinna* by Clark et al. (Clark et al. 2008a). This indicates that their physiological functions would be comparable between the species. Interpretations have the caveat that possible isoforms not found in the initial gene cloning could also contribute to the observed differences in the stress response.

Although showing acute up-regulation in the HSR and being grouped to stress-induced Hsps by sequence similarity, my measurements show that *hsp70B* is expressed in significant amounts under untreated control conditions, in all three geographically distant and ecologically different *Nacella* subpopulations examined (Fig. 3.3). I suspect this isoform to be routinely expressed in South American *Nacella* limpets. Splitting up Hsps into the two groups for constitutive and heat shock inducible tasks might be an inappropriate simplification in this case, and there is no physiological reason why chaperones that are

important in the unstressed status of an organism should not be further induced in the response to stress. In terms of total expression quantity of the investigated heat shock proteins, *hsp70B* is of primary importance in the acute stress response to tidal emersion. Its expression level during the response is about 4 times higher than *hsp70A* in the PM and PA_{sub} subpopulations (Fig. 3.2). Activation of *hsp70A* was higher in terms of gene up-regulation, because mRNA for this isoform was only present at very low levels in the limpets sampled before stress treatments. This agrees with the scheme in intertidal *N. concinna*, where *hsp70A* is highly up-regulated early after tidal emersion, while *hsp70B* shows a more moderate and prolonged response (Clark et al. 2008b). I thus interpret the A variant as a more short-term and/or specialized actor in the acute stress response upon air exposure, temperature elevation and possibly other stresses, not precociously activated in preparation of tidal emersion.

Patterns of *hsp70A* and *hsp70B* up-regulation were found to differ characteristically between the intertidal and the subtidal subpopulation at Punta Arenas, and between this location in the Strait of Magellan and the Northern Patagonian Puerto Montt population. In the following, I will analyze these differences and draw conclusions about the ecological role of the stress response.

4.3 Influence of intertidal zonation on the stress response

Near Punta Arenas in the Strait of Magellan, closely related *Nacella* limpets in an intertidal and a subtidal subpopulation were compared. In the intertidal specimens, up-regulation of *hsp70A* and *hsp70B* occurred later during air exposure and was between 2- and 10-fold lower in all treatments compared to the subtidal specimens. In the 6h treatments, *hsp70A* expression was higher, and in the 2h and 12h groups in the experiment at PA, *hsp70A* and *hsp70B* levels. Maximum levels were recorded after 6h of air exposure as opposed to 2h in subtidal animals. This correlates with the daily tidal emersion of 2-4h that the intertidal subpopulation undergoes at its position in the vertical gradient and shows acclimatization to intertidal conditions. Interestingly, differences in up-regulation were more marked in *hsp70A* than in *hsp70B*, and total *hsp70A* levels during the response are significantly lower in PA intertidal limpets than in subtidal animals, whereas *hsp70B* levels are equal.

Most studies on differences of the HSR in intertidal zones have focused on the variation in induction temperature. Higher temperature thresholds have been found in high intertidal molluscan populations than in low- and mid-intertidal zones, even after laboratory acclimation at a common temperature, indicating intrinsic fixation of this characteristic (Dong et al. 2008, Tomanek and Somero 1999, Sanders et al. 1991). Since the main trigger for activation of the HSR is the cellular amount of non-native proteins (Feder and Hofmann 1999), stress levels that are represented by temperature thresholds can be translated into

temporal stress limits that lead to sufficient protein damage to activate the HSR. I deduce that acclimatization to the intertidal habitat leads to a delayed stress response in the intertidal *N. magellanica* as compared to its subtidal congener at the same location.

In investigations of field Hsp levels, higher expression of constitutive Hsps has been found in high intertidal subpopulations of the limpet *Lottia* (Dong et al. 2008) and the mussel *Mytilus* (Place et al. 2008, Halpin et al. 2002). Constitutive Hsp field levels remain comparatively higher in low-intertidal snails of the genus Chlorostoma after transplantation to the mid-intertidal (Tomanek and Sanford 2003). In the present study, average *hsp70B* and *hsc70* expression levels in field-acclimatized control animals appeared to be slightly higher in intertidal limpets than in subtidal specimens from the same location, but variation was high and the difference, although plausible, was statistically not significant. In comparison, constitutive expression discrepancies in orders of magnitude have been reported for *N. concinna* and *Mytilus* (Clark et al. 2008b, Place et al. 2008). Although limpets of the subtidal group were not from the deeper subtidal zone that could be regarded as definitely free of tidal influence, they should not experience regular emergence stress. Thus, I find no support for a connection of constitutive Hsp expression levels with intertidal zonation. It can be concluded that not only expression levels, but also the timeframe of the heat shock response varies with acclimatization to the natural habitat, leading to a later or slower onset of the response of sub-polar *Nacella* limpets at a lower position in the intertidal zone, and that constitutive Hsp expression is probably not accountable for the difference. Since constitutive Hsp70 expression of the control animals was not significantly higher in the intertidal population, I hypothesize that intertidal *Nacella* limpets are less stressed by regular tidal emersion because they possess other mechanisms for stress compensation. The different shape of the shells could be partly responsible. Intertidal *N. magellanica* have higher shells, which leads to lower contact zone to volume ratios than the flatter shells of *N. deaurata* (Fig. 3.1). The higher volume of shell water could buffer temperature increases, and the higher surface area could increase wind cooling, mitigate emersion stress and reduce the need for protein protection via the heat shock response.
It may be noteworthy that some indication for differential regulation under tidal emersion can also be seen in the candidate reference genes. A down-regulation to 25% of Histone H3 expression was measured in the PM 12h animals, and non-normalized β-actin levels are reduced to 50-70% in subtidal PA animals of the 6h and 12h treatments. In other treatments from PA_{sub} and PM, a trend towards down-regulation of these two genes can be suspected, but is not significant. As both genes produce proteins involved in cell structural tasks, this could be an indication for a stop in growth and cell division in the foot muscle tissue of all but the high-intertidal PA limpets. This would have to be tested in another experiment, and possibly by finding more stable reference genes.

In summary, the heat shock response observed in intertidal *Nacella* was weaker than in its subtidal relative, and its temporal pattern correlates with the usual emersion at

its preferred tidal position. This difference was especially pronounced in the expression of *hsp70A*, and could be intrinsically fixed in the two different subpopulations. The intertidal *N. magellanica* and the subtidal *N. deaurata* limpets at the experimental site near Punta Arenas have been designated as two different *Nacella* species, but their genetic divergence is low (González-Wevar et al. 2010). The differential adaptation to intertidal exposure could be an essential characteristic of the two main species groups in Patagonia, and I will elucidate this issue below by analysis of the Hsp70 gene diversity (cf. 4.5).

4.4 Biogeographic comparison of the stress response

Maximum inducible HSP expression levels in the Puerto Montt population have been on average 3-4 times higher than in both subpopulations from the Strait of Magellan, whereas constitutive chaperone levels are similar. This correlates with higher air temperatures on the day of the experiment: PM animals had been exposed to an average air temperature of 19°C when they showed their maximum HSP expression after 6h of treatment, while temperature in PA was only 16° on average (Fig. 3.4). Both temperature regimes, with ranges of 15-22°C (PM) and 13-17°C (PA), are representative for the climatic conditions experienced by the limpets on an autumn day in their respective locations . *hsp70A* and *hsp70B* expression are not only higher, but also show markedly less inter-individual variation at PM, which is a further indication for a pronounced heat shock response. The PM locality is among the northernmost points in the distribution of *Nacella* in South America and the two locations sampled in this study are separated by a large geographic distance. This suggests that intertidal animals in a temperate habitat produce higher levels of Hsps than their subpolar congeners, as a result of the necessity to cope with the more pronounced increase in temperature that they undergo upon tidal emersion. The size differences further illustrate this point: PM specimens were significantly smaller than both PA subpopulations, implying that investment of a larger amount of their cellular energy for stress compensation could restrict shell growth in these ectotherms.
Comparisons of Hsp70 levels in other intertidal invertebrates have already shown that in general, organisms are more stressed towards the edges of their distribution range (Osovitz and Hofmann 2005, Tomanek 2008, Sorte and Hofmann 2004). Especially at the edges of geographical distribution, habitat microstructure plays an additional role (Place et al. 2008, Helmuth et al. 2006).
In spite of acclimatization and temperature regimes during emersion being not the same for the two populations in our study, laboratory acclimation studies showed that the observed differences in Hsp induction and thermotolerance between geographically distinct populations of intertidal species animals are intrinsically fixed (Fangue et al. 2006, Tomanek and Somero 1999). This suggests that the physiological capability for a high stress response can be based on evolutionary adaptation and may limit colonization of stressful habitats,

and contribute to setting the geographical range boundary of an intertidal species.

4.5 Allelic differentiation in the *hsp70A* locus

It is still unclear to what extent the two *Nacella* species in this study are reproductively isolated. The observed expression differences between the intertidal and subtidal limpets could be the result of phenotypic plasticity, or be caused by a differential distribution of genotypes. To further investigate this aspect, I assessed the genetic variation in the Hsp70 genes themselves. In the partial *hsp70A* gene sequences, two different groups were identified that were unambiguously supported by 55% of the polymorphic sites, and I assigned 44 samples as heterozygotes, assuming evolutionary linkage of these sites. The haplotype network of *hsp70A* separates A1 and A2 on a 95% parsimony level. Under the premise that these represent two distinct allele groups of a single *hsp70A* locus, I examined the divergence in this gene. In this paragraph, I will analyse the results under the hypothesis of neutral evolution, and indications against this null hypothesis will be provided in the next paragraph.

In the experimental population at Punta Arenas, the two species showed a clear genetic differentiation in the distribution of the two allele groups A1 and A2, despite their small spatial distance of just a few meters. Further sequences were obtained from genomic DNA samples in the Magellan Province and confirmed this picture. Pairwise F_{st} estimates, based on the distribution of A1 and A2 and 12 further polymorphisms, were 0.13–0.86 between subpopulations of *N. magellanica* and *N. deaurata* among all sampled sites. High values of 0.69–0.76 between species were obtained at three locations in the Central and Western Strait of Magellan and in the Beagle Channel, where both species have been sampled at the same site. This discovery provides strong support for the existence of genetic divergence in the *hsp70A* locus between the two most abundant limpet species of the Magellan Province. In this region, *Nacella* is morphologically diverse, but recent molecular genetic analyses detected only a very low degree of genetic differentiation. The mitochondrial markers COI and Cyt b failed to distiguish *N. magellanica* and *N. deaurata*, and the question was raised if their clear morphological differences may be solely the result of phenotypic plasticity, and they could rather be regarded as ecotypes (De Aranzamendi et al. 2009, González-Wevar et al. 2010). The discrepant distribution in the *hsp70A* locus indicates that genetic differentiation does exist, and the additional microsatellite data confirm a significant divergence between *N. magellanica* and *N. deaurata* populations.

The four sampled *N. magellanica* populations at the Pacific coast are fixed for the A1 allele group, while in the localities further into the Strait of Magellan, proportions of A2 increase progressively towards the Atlantic opening. An analysis of this distribution has to be performed on the background of the oceanographic and climatic history of the

region. During the Last Glacial Maximum, no passage through the Strait of Magellan was possible for marine animals because of the lowered sea level, while the Pacific coastline and the Western half of the Strait were covered by ice (Mcculloch and Bentley 1998), which would make this region largely uninhabitable for intertidal limpets. Around 10,000–8,000 years ago, temperatures rose, ice retreated and sea level increased, opening up the passage from the Atlantic side first (McCulloch and Davies 2001, Porter et al. 1984). It is thus probable that this channel represents a secondary hybrid zone, where *Nacella* populations from glacial refugia at opposite sides met after retreat of the ice (Leese et al. 2008). In isolated refugia, populations can evolve independently, leading to allelic divergence, which is increased by founding events upon re-colonization of habitats before their secondary contact (Hewitt 2000).
N. magellanica and *N. deaurata* are reported to be widely distributed in Patagonia, and are suspected to have a planktonic larval stage of 3-4 weeks (González-Wevar et al. 2010). The two *Nacella* species were found in sympatry at three of the sampling sites, and strong semidiurnal currents are present in the Strait of Magellan (Medeiros and Kjerfve 1988). Therefore, equal mixing of alleles would have been expected in today's *Nacella* populations, unless there is a high degree of reproductive isolation between the original lineages or the analysed locus is located in a genomic region that is under divergent selection (Mallet 2005).
It has to be noted that the allele groups do not correlate perfectly with the identification of *N. magellanica* and *N. deaurata* based on morphological and confirmed by microsatellite data. It is thus possible that either reproductive isolation is high, but not complete, or that lineage sorting in the *hsp70A* locus is incomplete. As a first conclusion of the analysis of allelic diversity under the hypothesis of evolutionary neutrality, the clear divergence in the *hsp70A* locus could be caused by differential fixation in the evolutionary lineages and then would be an indicator of a high degree of reproductive isolation between *N. magellanica* and *N. deaurata*. Experimental mating studies among the two *Nacella* species would be needed to test if the genetic divergence in this locus can be explained by reproductive isolation.

4.6 Indication for adaptive divergence of *hsp70A*

The strong disproportions of the A1 and A2 allele groups among the sampled *Nacella* subpopulations could not only be a result of their genetic history, but could also point to present-day differences in adaptation to environmental conditions. Estimates of allelic divergence in the microsatellite markers can be taken as a neutral baseline of genetic differentiation (Hemmer-Hansen et al. 2007). The neutral differentiation was standardized to the maximum value possible, to account for differences in the expected levels of homozygosity between the two marker types (Hedrick 2005). Divergence in the *hsp70A* locus was

very similar to the standardized F'_{st} levels in some of the pairwise comparisons of subpopulations, but considerably higher in others: The differences in the F_{st} estimates of the two markers are 0.46-0.52 between *N. magellanica* and *N. deaurata* at their different tidal positions within the three shared sampling sites, and 0.25-0.29 between the *N. magellanica* populations in the Eastern Strait of Magellan (Bahía Gregorio and Punta Catalina) and the sites at the Pacific coast. This clear disparity in distribution suggests that the *hsp70A* locus itself or a closely linked locus has undergone adaptive divergence.

This observation is supported by changes on the protein level. Sequence analysis of the three cytosolic Hsp70 isoforms in *N. magellanica*, *N. deaurata* and *N. concinna* shows that the heat shock proteins are highly conserved among the three *Nacella* species, although the Magellanic limpets have split from the Antarctic lineage approx. 9 Ma ago (González-Wevar et al. 2010). Against this background, the seven amino acid differences in the 960bp partial sequence of the two Patagonian *hsp70A* allele groups represent a considerable differentiation. On the other hand, within the A1 and A2 allele groups assigned in the 693bp alignment, there are no amino acid substitutions, but 12 silent polymorphisms. This indicates that non-synonymous mutation is constrained and purifying selection has been acting on this gene in the recent evolutionary past after divergence of the two allelic lineages (Nielsen 2001). With complete sequence information and an analysis of more samples from *N. concinna* or another close relative, this reasoning could be supported by codon substitution models and statistical comparisons of divergence and polymorphism between the three species, and it might be possible to distinguish between purifying or diversifying selection (Bustamante et al. 2005, Yang et al. 2000).

Since the intertidal and subtidal *Nacella* species in my study show preference to distinct tidal positions, disagree in the expression level of *hsp70A* during the acute heat shock response upon experimental air exposure, and show increased genetic divergence and indication for selection in the *hsp70A* locus, it seems plausible that *hsp70A* is involved in a differential tolerance of tidal emersion stress in *N. magellanica* and *N. deaurata*. Furthermore, the major shift in *hsp70A* allelic distribution in *N. magellanica* populations in the Central Strait of Magellan conincides with differences in reported tidal ranges, which increase from west to east. While on the Pacific side of the strait, spring tide ranges attain a moderate 1.2m, near the Atlantic opening, mean tide ranges reach 8.4m (Medeiros and Kjerfve 1988). Since *hsp70A* is not constitutively expressed in the analysed samples, it seems more likely that air exposure duration and air temperature would contribute to increased divergence in this heat shock protein gene, rather than water-borne stressors like water temperatures, salinity or toxicants.

If fostered by more empirical evidence that lies beyond the scope of my work, a connection between the sequence and the expression pattern of the *hsp70A* genes could be established. Firstly, my assignment of polymorphisms into the two allele groups would have to be

confirmed by molecular cloning of heterozygous samples and sequencing of the complete coding region, and then compared with differences in regulatory regions. Secondly, samples from more populations, especially of *N. magellanica* along the Atlantic coast, could elucidate whether allelic distribution shows stronger links to biogeographic history or to present environmental parameters. Finally, a differential fixation of the *hsp70A* alleles in *Nacella* populations could be confirmed by experiments involving replanting of subtidal populations to a higher intertidal zone or by cultivation under different stress conditions, if possible over multiple generations.

4.7 Conclusions, implications and speculations

The classical heat shock response has been proposed as an integrative measure of physiological stress, which could be used to assess to which extent organisms are affected on the long term by rising temperatures, i.e. a biomarker for climate change at the individual and community levels (Halpin et al. 2002, Clark and Peck 2009b, Tomanek 2010). The results of my study provide examples for problems associated with this project, but they also demonstrate the potential for a prognostic value that can be obtained by linking Hsp70 expression to genomic information.

High individual variations were present in expression of the heat shock genes under emersion stress. These could be lowered by suitable data handling, but even with optimal normalization, some biological variation will remain. The heat shock response is highly influenced by the stress history of each individual, which is dictated by a microhabitat structure that often is not visible upon sampling (Sagarin and Somero 2006, Halpin et al. 2002). Even in animals acclimated to a constant laboratory regime, variations in the response are often very high (Clark and Peck 2009b). As the comparison of intertidal and subtidal specimens in our study shows, the plasticity of the heat shock response has a temporal component. Due to the steep gradient of induction, a small shift in onset time of the response is sufficient to produce high variability at a discrete time point when samples for gene expression measurements are taken, even if the overall expression patterns would be identical. Possibly, peak protein or transcription levels of the reaction are less plastic, but as it is not possible to sample the same individual for repeated times, this variability presents a problem for the use of the heat shock response as an ecological stress indicator. Constitutive HSP expression levels are also variable in my study, especially in the PM population and in PA intertidal limpets. This effect may be connected with the high stress fluctuations that intertidal animals experience. But if this plasticity has a genetic basis, as the differences in individual variation and response strength between the intertidal and the subtidal subpopulation in my study suggest, this disadvantage might be turned into an advantage when it comes to following big-scale changes in species dis-

tribution and population dynamics. As high levels of Hsp synthesis signify energetic costs and potential cytotoxic effects to the organism, it is not likely that individual variations only reflect a relaxed environmental selection pressure on this trait. Rather, they could be reflecting differences in gene regulatory regions or physiological, morphological and behavioural compensation mechanisms. If these were hereditary, then the HSR could still be a good negative indicator.

The allelic distribution of *hsp70A* could be an imprint of the biogegraphic history of *Nacella* in the Magellan region, or be directly caused by different environmental parameters. *N. magellanica* could have evolved physiological, morphological and behavioural adaptations to intertidal exposure that its sister species doesn't possess, and thus reduce the need for protection by *hsp70A*. Alternatively, its *hsp70A* alleles could be more effective in mitigating stress, and therefore lower expression levels would be needed. These adaptations could convey an advantage by facilitating access to energy-rich food resources. Indeed, the intertidal limpets grow significantly larger than the subtidal specimens. On the other hand, high and stable levels of Hsp expression in *Nacella* at the location near its Northern distribution edge in Puerto Montt may signify that the species is prevented from colonizing warmer habitats by the increasing stress upon tidal emersion. So, *Nacella* in habitats like Puerto Montt might be threatened most by an increase of water and air temperatures. On the other hand, the adaptation to a stress extreme that this population has undergone might be a source of adaptive alleles to congeners in more central parts (Davis and Shaw 2001) and help to colonize intertidal habitats that become available.

Although the *hsp70* genes are a plausible candidate for adaptive divergence, because of their generally high conservation, to my knowledge, few studies have attempted to link Hsp70 sequence data to expression levels (Yokoyama et al. 2006, Fangue et al. 2006). In my study, relative sequence conservation in the homologous regions of the *Nacella* Hsp70 genes correlates with their constitutive expression levels. Since *hsp70A* is the only chaperone in my assay that is only expressed at background levels in all control animals, it is a possible explanation that this isoform has originated from a duplication of *hsp70B* many million years ago, experienced regulatory changes and has undergone adaptive evolution in the coding sequence because of relaxation of the constraints posed by a constitutive role in the organism.

In most ecophysiological publications using gene expression quantification by RT-qPCR, control expression levels are not reported, because the method is not yet standardized enough to allow comparison of absolute levels between laboratories, and different efficiencies in RT can prevent comparison between genes. Nevertheless, genes with a high degree of sequence conservation, like in the Hsp70 gene family, could still be compared without reference to a control group. Thus, by normalization to one or two highly conserved and evaluated housekeeping genes, total Hsp expression levels in the field could be compared between populations and between related species. This information could be highly useful

in analyzing the ecological role and plasticity of Hsp70 expression, the participation of the separate isoforms in the stress response, and provide clues for the potential sequence evolution of the paralogous genes.

If the heat shock response is influenced by other factors than just temperature, this does not reduce its usefulness, but can speak for its quality as an integrative biomarker. E.g., additional anthropogenic stressors such as toxicants have been shown to elicit the HSR (Sanders 1993), and these stressors also increase the danger of damaging stress levels to organisms, and thus the potential adverse effect of high temperature. Adaptation to stress is an essential part of life and an ability that will be in demand in areas affected by climate change (Bijlsma and Loeschke 2005), and the heat shock response may be a suitable marker to track the developments in the planet-wide selection experiment that humans are conducting (Reusch and Wood 2007).

Many population reactions that are attributed to adaptation might in reality be results of phenotypic plasticity (Gienapp et al. 2008), and the mechanisms underlying plasticity and genetic fixation will be key questions of the assessment of adaptation to climate change (Visser 2008). Thus, the heat shock response might indeed be a good model system, not only on the physiological, but also on the evolutionary level. But to me, it has also become clear that it requires substantial effort to assess all factors that may influence expression levels, and fascinatingly many more to follow the propagation of the Hsp70 genes along the evolutionary lineages.

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Appendix

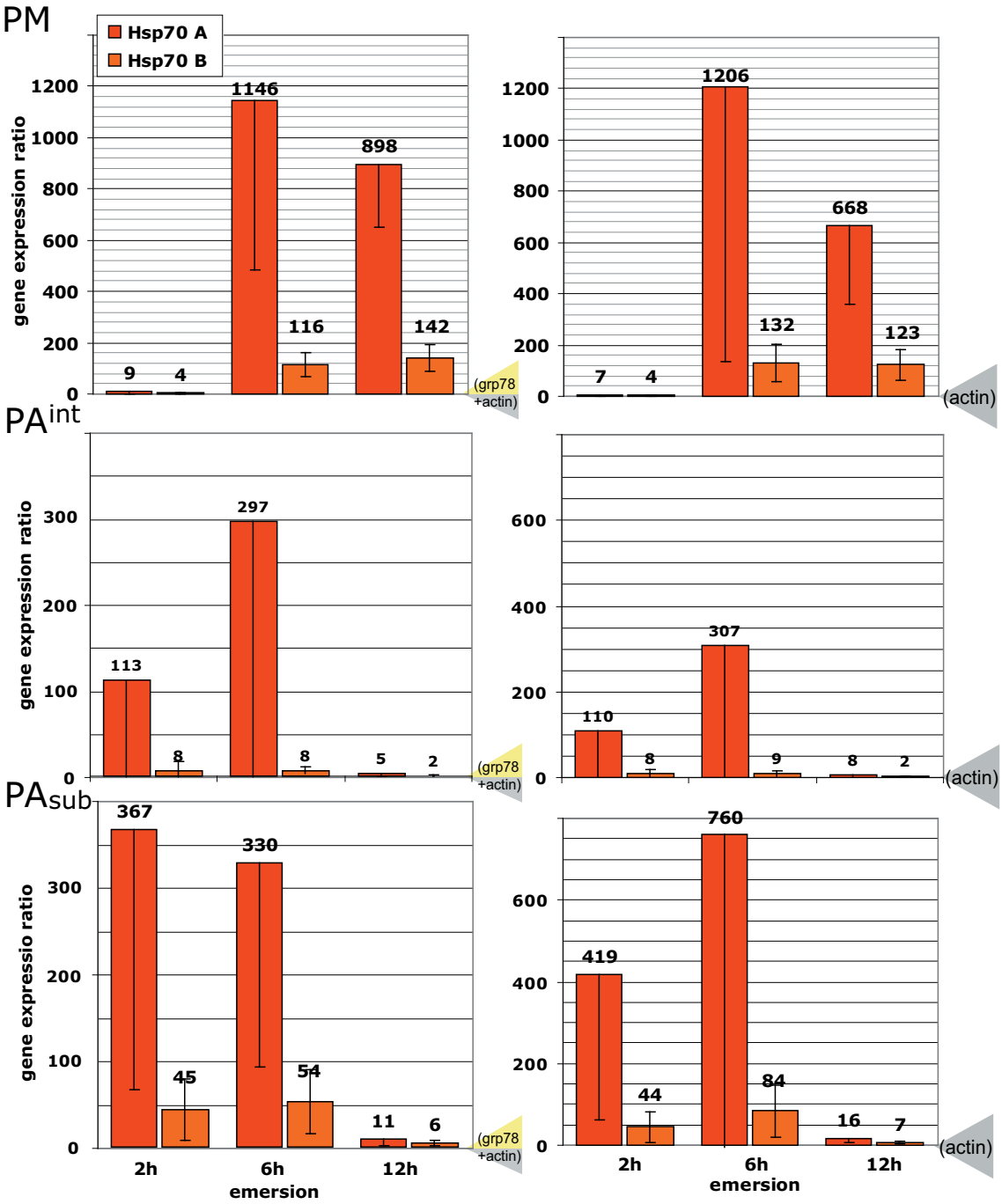

Figure 4.1: Up-regulation of *hsp70A* and *hsp70B* expression in the emersion experiments, normalized to Grp78/β-actin (left) and β-actin (right), calculated by standard curve quantification. Mean values of relative expressions compared to untreated control animals for the three subpopulations Puerto Montt (PM), Punta Arenas intertidal (PA_{int}) and subtidal (PA_{sub}). N=6 for each group except N=4 for 6h-PA_{sub}. Standard deviations (biological variation) are symmetrical but only depicted in one direction for *hsp70A*.

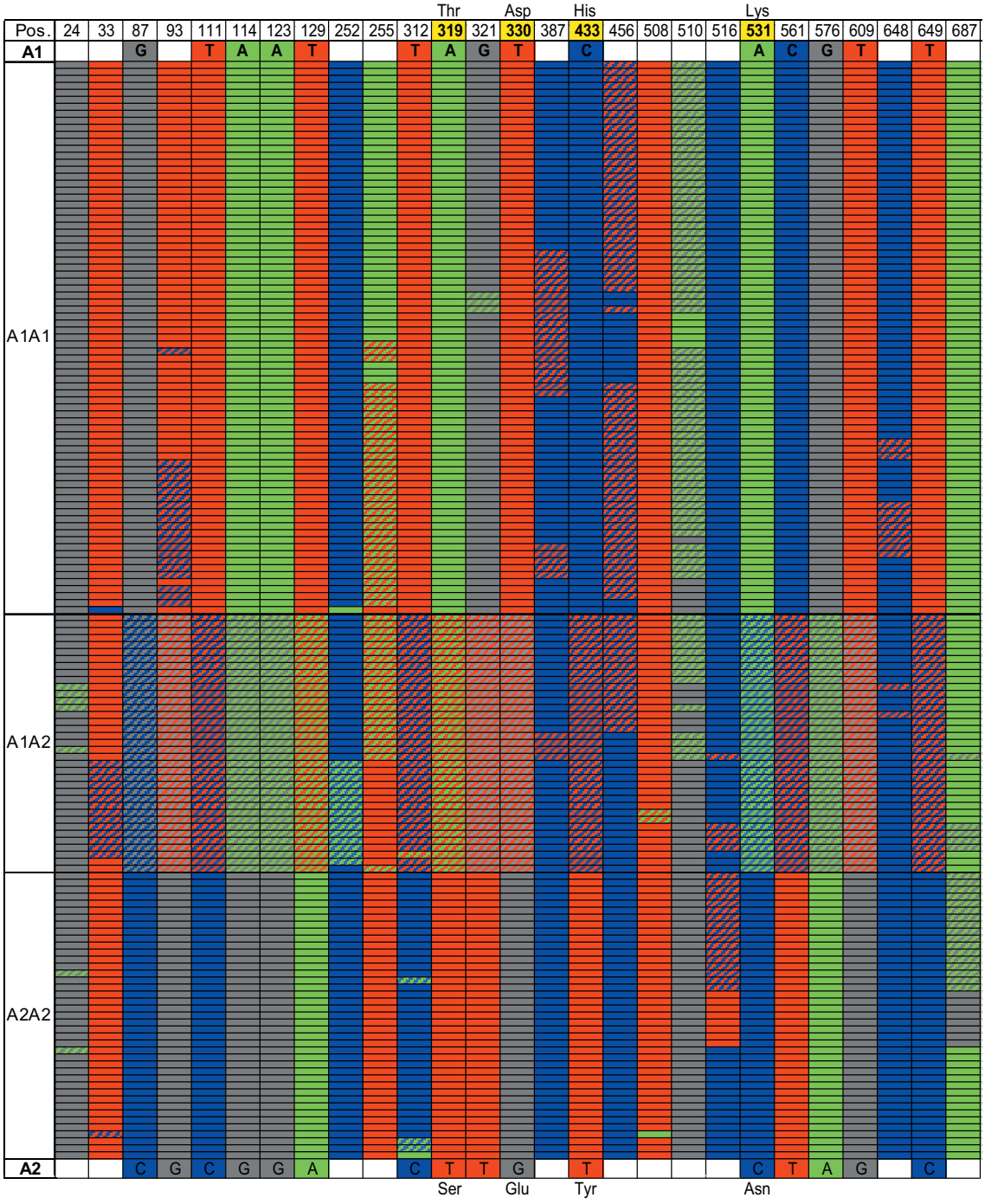

Figure 4.2: Distinct *hsp70A* allele groups in *N. magellanica* and *N. deaureata*. Columns are parsimony-informative sites in the 700bp alignment of *hsp70A* DNA sequences, each row represents one of the 161 sequences, colours mark bases A, C, T, G at the respective positions and shaded cells have both of the two alternative bases. 15 sites follow a clear distribution pattern, four sites lead to amino acid substitutions (position numbers marked yellow, three letter codes). Samples have been grouped into homozygotes and heterozygotes accordingly.

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