Fungal and cyanobacterial gene expression in a lichen symbiosis: Acclimatization and adaptation to temperature and habitat

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90 ECTS thesis submitted in partial fulfillment of a
Magister Scientiarum degree in Molecular Biology

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Abstract

How do organisms react to a warming environment? How do such environmental changes affect species interactions? These are key questions in times of global warming. One strategy of coping with environmental changes is acclimatization. Therefore, species evolved complex molecular stress-response mechanisms including heat-shock systems (protein repair) and DNA repair pathways.

Here, we analyzed candidate gene expression in the lichen fungus *Peltigera membranacea* and its cyanobacterial *Nostoc* symbiont, a key player in carbon and nitrogen cycling in terrestrial ecosystems at northern latitudes. Do increasing temperatures cause a stress response reflected in expression levels of selected stress-response genes and do stress response patterns differ between coastal and inland habitats? As mutualistic symbioses, lichens offer the possibility of analyzing molecular stress responses in a particularly tight interspecific relationship.

Using real-time PCR quantification of 38 transcripts, differential expression was demonstrated for nine cyanobacterial and nine fungal stress response genes (plus the fungal symbiosis-related *lec2* gene) at 15°C and 25°C vs. 5°C indicating temperature stress for both symbionts. Principle component analysis (PCA) revealed two gene groups differing in temperature response patterns. Whereas a set of cyanobacterial DNA repair genes and the fungal *lec2* (PC1 group) showed a strongly correlated expression drop at 15°C vs. 5°C, most fungal candidates (PC2 group) showed increased expression at 25°C vs. 5°C. Furthermore, PC1 genes differed more strongly in temperature response along an elevation gradient than between habitat categories. The correlated downregulation of *lec2* and cyanobacterial DNA repair genes suggests a possible interplay between the symbionts warranting further studies.

Útdráttur

Hvernig bregðast lífverur við hlýnandi loftslagi? Hvernig hafa umhverfisbreytingar áhrif á samspil tegunda? Þetta eru lykilspurningar varðandi áhrif loftslagsbreytinga. Ein leið til að fást við breytingar í umhverfi er langtímaaðlögun. Vegna þessa hafa lífverur m.a. þróað flókin streituviðbrögð á sameindasviðinu, svo sem hitalosts viðbrögð (viðgerð á próteinum) og viðgerðir á erfðaefninu.

Í þessu verkefni hafa viðbrögð valinna gena í fléttusveppnum *Peltigera membranacea* og *Nostoc* blágrænbakteríusambýlungi hennar, sem gegnir mikilvægu hlutverki í hringrásum kolefnis og niturs á norðurslóðum. Veldur hitastigsaukning streituviðbragði sem kemur fram í tjáningu valinna gena sem tengjast streituviðbrögðum, og er munur á viðbrögðum flétta nærri sjó og langt inni í landi? Þar sem fléttur eru sambýlislífverur, þá gefst tækifæri til að skoða streituviðbrögð í sérstaklega nánu samfélagi tveggja ólíkra lífverugerða.

Tjáning 38 gena var mæld með rauntíma PCR aðferð, og fannst breytileg tjáning hjá níu genum blágrænbakteríunnar og níu streitutengdum sveppsins (auk samlífis tengda gensins *lec2*) við 15°C og 25°C miðað við 5°C, sem bendir til hitastreitu viðbragða í báðum sambýlungunum. Fjölbreytugreining (PCA) sýndi tvo hópa gena með ólíka svörun við hitaáreiti. Annars vegar sýndu DNA gen tengd DNA viðgerðum í blágrænbakteríunni og sveppgenið lec2 (PC1 hópur) svipað fall í tjáningu við 15°C miðað við 5°C, en hins vegar sýndu flest sveppgenin (PC2 hópur) aukna tjáningu við 25°C miðað við 5°C. Jafnframt sýndi tjáning PC1 genanna meiri samsvörun við hæð en milli megin vistgerða. Samsvörun í tjáningu *lec2* og DNA viðgerðargena blágrænbakteríunnar gæti bent til samspils milli sambýlunganna sem vert er að skoða betur.

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Abbreviations

ANOVA	analysis of variance
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CO_2	carbon dioxide
DNA	desoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ESR	environmental stress response
HPLC	high performance liquid chromatography
HSP	heat-shock protein
H_2O	water
IAA	indole-3-acetic acid
kDa	kilo-Dalton
O_2	oxygen
PC	principle component
PCA	principle component analysis
PCR	polymerase chain reaction
ppm	parts per million
RNA	ribonucleic acid
RQ	relative quantity (relative expression)
RT-qPCR	quantitative real-time PCR
SNP	single-nucleotide polymorphism
STRE	stress-responsive element
TBP	TATA-binding protein

Acknowledgements

For giving me the great opportunity to perform this project as part of my master studies in molecular biology at the University of Iceland, I want to thank my supervisors **Prof. Dr. Ólafur S. Andrésson** and **Dr. Silke Werth**. Thank you for guiding me through all the practical and theoretical work of the project! I truly enjoyed working in your research group and gained a lot of useful experience for my future. Furthermore, I want to thank my supervisor **Dr. Arnar Palsson** especially for his patient help throughout the statistical data analysis for the project and **Prof. Dr. Zophonias O. Jónsson** for his productive input during the planning and the conduction of the project.

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1 Introduction

In times of climate change and global warming it has become a key question throughout different research fields how changing environmental conditions could affect species within their habitats. A reduction in biodiversity can negatively impact ecosystems and therefore also the benefits and services they provide for human society (Schröter 2005, Burkle et al. 2013).

In the past decades there have been numerous reports on rapid climate changes on global scales (Diffenbaugh and Field 2013, Penuelas et al. 2013). Phenomena such as increasing temperatures and increasing CO_2 concentration have an impact both on terrestrial and marine ecosystems (The Royal Society 2005, Diffenbaugh and Field 2013, Penuelas et al. 2013). Species have to cope with environmental changes occurring much more rapid than in the past centuries. During the short time period from the beginning of the industrial revolution the atmospheric CO_2 level has risen from 280 ppm up to the current 394 ppm (Penuelas et al. 2013). In a variety of marine ecosystems increased CO_2 partial pressures have a strong negative impact on calcifying organisms (The Royal Society 2005, Albright et al. 2010) as well as non-calcifying species by affecting growth, physiology, respiration and metabolism (Pörtner et al. 2004, Pörtner 2008).

Another aspect of climate change are increasing temperatures, causing major changes both in marine and terrestrial ecosystems by affecting species on many levels from metabolism and growth to general species interactions and ecosystem productivity (Penuelas et al. 2013). Studies on a variety of terrestrial species (e.g. butterflies, birds, higher plants) have shown that increased temperatures affect breeding, blooming (e.g. of the lilac *Syringa vulgaris* in the western USA) and the length of growth seasons (Parmesan 2006). Furthermore, temperature shifts can lead to asynchrony in species abundance and therefore influence predator-prey, insect-host and other interactions. Studies on butterflies have shown that asynchrony between butterflies and their host plants can result in population extinctions (Parmesan 2006). Moreover, phenomena such as the invasion of foreign species into new habitats are often associated with climatic shifts (Landschoff et al. 2013). Thus, species and their ecosystems can be affected in various ways by increasing temperatures, which can further lead to major range shifts affecting ecosystem goods and services (Penuelas et al. 2013). For that reason, research has focused on whether and in which ways species can adapt to environmental changes and thus avoid extinction (Parmesan 2006). However, environmental changes due to climate change might not have the same effect on species in all types of habitats. For marine habitats it has been suggested that both changing CO_2 levels and temperatures might have different effects on species originating from naturally variable coastal habitats than deep-sea habitats with generally very stable temperature, CO_2 and O_2 conditions (Pörtner et al. 2004, Pörtner 2008, Melzner

et al. 2012). Terrestrial environments often differ in the magnitude of external factors such as temperature. Whereas open areas in the interior can show high variability in terms of temperatures, areas at the coast or in forests are characterized by less temperature fluctuation. Studies on lichens have shown that epiphytic floras are altered more rapidly in response to climate change in open rural areas compared to forested areas (Aptroot and van Herk 2007). Therefore, the effect of global warming on a species can be rather complex, since different populations of one species located in habitats with different temperature regimes might be affected differently by global warming.

Under environmental stress there are several options enhancing survival of species. One possibility is migration to more favorable habitats. In the Netherlands for example, lichen species associated with warmer temperatures have significantly increased in abundance whereas cold-temperature species have either decreased or disappeared (Aptroot and van Herk 2007). In contrast, acclimatization is a way of coping with environmental changes without necessarily leaving the habitat. Acclimatization to local conditions is a general phenomenon based on phenotypic plasticity, which describes the capacity of a single genotype to develop variable phenotypes in different environments manifesting as changes in biochemistry, physiology, morphology, behaviour and life history (Whitman and Agrawal 2009). Acclimatization can take place within minutes or hours but can last for weeks or months (Rachmilevitch et al. 2008, Odsbu and Skarstad 2014, O Leyva-Perez et al. 2015). However, in contrast to genetic adaptation acclimatization is more of a short-term process, which is usually reversible and the result of which is not necessarily passed to the next generation. Therefore, local adaptation is another survival strategy with an underlying genetic base, which is acting on larger time scales than acclimatization. If enough individuals of a species´ population are able to cope with environmental change, the population will have a chance to survive. Adaptation evolves by natural selection, since those individuals of a population that have a better ability to cope with a changing environment do better and have more offspring (Davis 2005, Aitken et al. 2008, Manel et al. 2010). At the genetic level, the occurrence of single-nucleotide polymorphisms (SNPs) in genes functionally involved in coping with specific environmental conditions can be an indication of adaptation due to selection pressure. In a variety of different organisms including fungi, adaptation to more demanding conditions has been investigated using SNPs in candidate genes taking part in environmental stress response (ESR) processes such as heat-shock or DNA repair (Gasch 2007). Another process important in adaptation is gene flow between populations, a key mechanism for the introduction of new genetic variants into populations (Kawecki and Ebert 2004). Beneficial gene mutations can spread rapidly within and between populations through high gene flow and increase fitness and survival in a changed environment (Dalziel et al. 2009).

Both acclimatization and adaptation are key mechanisms in coping with a changing climate. Physiological flexibility, driven by organisms´ acclimatization to local site conditions, plays an important role for species to successfully cope with environmental variability (Davis 2005, Aitken et al. 2008, Manel et al. 2010). The ability of species to

cope with various environmental stresses is of exceptional importance for the long-term persistence of their populations in a given environment. When enough individuals of a population have the ability to adjust their life processes to changed environmental conditions, populations are able to persist. One mechanism underlying phenotypic plasticity is the environmentally induced alteration of gene expression resulting in switches at the physiological level may enable species to tolerate changed environments by allowing them to survive stressful environmental conditions. At the molecular level, both prokaryotes and eukaryotes have developed complex environmental stress-response (ESR) mechanisms including a variety of pathways in order to react to external stress due to e.g. drought or heat (Apte et al. 1998, Plesofsky-Vig and Brambl 1998, Young 2001, Enjalbert et al. 2006, Gasch 2007, Zahrl et al. 2007, Sato et al. 2008, Che et al. 2013, Pasqua et al. 2013). As studies on plants and fungi have shown, drought can induce a number of cellular stresses including oxidative and osmotic stress, hyper-ionicity and protein misfolding. For coping with cellular stress, three main stress-response pathways play a major role including osmoregulation (modulation of intracellular ion concentration), antioxidation (scavenging reactive oxygen) as well as DNA and protein damage repair (Wang et al. 2015). It has been suggested that specific stress response programs can be combined to a global core stress response conserved throughout prokaryotes and eukaryotes, which is commonly activated in a variety of environmental stresses (Lopez-Maury et al. 2008). In fungi (*Saccharomyces cerevisiae, Schizosaccharomyces pombe*), a great number of candidate genes involved in environmental stress response have been identified (Gasch 2007). Under stress, common stress response signaling pathways including e.g. the cAMP signaling pathway, mitogen-activated protein kinases or the Hog1 pathway are activated, which mediate the transcriptional upregulation of ESR candidate genes (Wang et al. 2015). In the Hog1 pathway in *S. cerevisiae*, which developed orthologously to the Sty1 pathway in *Sz. pombe* (Gasch 2007), the occurrence of environmental stress (heat, osmotic or oxidative stress) leads to a repression of two-component regulatory histidine kinase Sln1. Sln1 is responsible for the repression of Ssk1 and therefore prevents Hog1 activation in non-stress conditions. The occurrence of environmental stress such as heat, osmotic or oxidative stress leads to a repression of Sln1 and therefore activates Hog1 through Ssk1 and further Ssk2 and Ssk22 (Gasch 2007). Additionally, phosphatases like Pyp1 and Pyp2 play a role in Hog1 repression under normal conditions. Hog1 further activates the expression of the transcriptions factors Msn2 and Msn4 (Rep et al. 2000, Gasch 2007, Liu et al. 2013), which are responsible for activating the gene expression of various stress response genes (heat shock genes, DNA repair genes) containing stress response elements (STREs)(Martínez-Pastor et al. 1996). As members of the heat shock gene family, Hsp genes have the ability to repair proteins denatured or misfolded due to external stress.

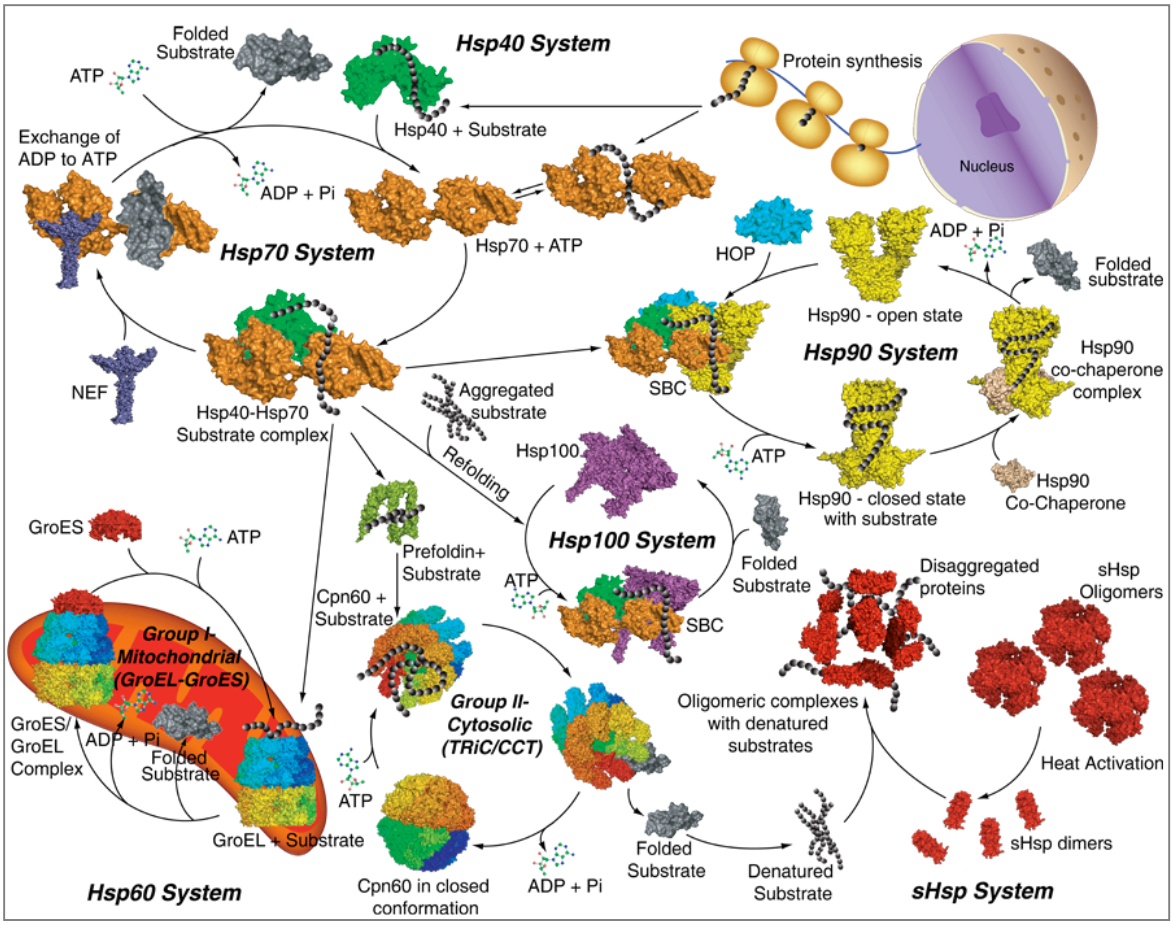

Figure 1: Hsp protein family network required for maintenance of cellular protein quality (http://pdslab.biochem.iisc.ernet.in/hspir/img/indexfigure.png, 28.05.2015)

External stress such as drought or heat can induce cellular damages at the protein level. There are many heat shock pathways (Fig. 1), highly conserved throughout both prokaryotes and eukaryotes (Langer et al. 1992, Wiech et al. 1992, Schüller et al. 1994, Hartl 1996, Mendoza et al. 1996, Goloubinoff et al. 1997, Plesofsky-Vig and Brambl 1998, Melkani et al. 2005, Enjalbert et al. 2006, Che et al. 2013, Pasqua et al. 2013, Rajaram et al. 2014). In the Hsp60 system, the 60 kDa chaperone GroEL forms a protein barrel structure with the GroES chaperone, in which denatured or misfolded protein substrates can be refolded in the correct way in an ATP-dependent process (Goloubinoff et al. 1997, Melkani et al. 2005, Zahrl et al. 2007, Pasqua et al. 2013, Rajaram et al. 2014). The Hsp40/Hsp70 complex in conjunction with the Hsp90 system contributes to protein damage repair of proteins at a later stage of folding (Fig. 1) (Wiech et al. 1992, Young 2001, Wang et al. 2007).

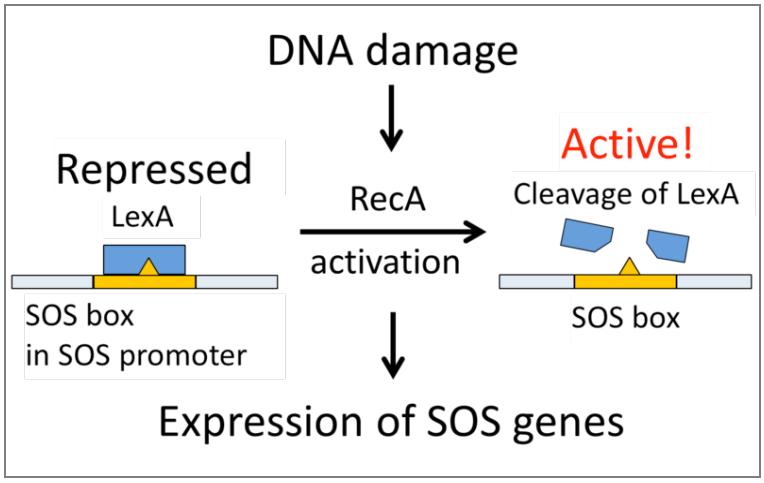

Figure 2: Interplay between RecA and transcription repressor LexA in the regulation of the SOS DNA damage response. In presence of single-strand DNA, expression of SOS genes is activated by RecA-mediated cleavage of LexA. (http://2012.igem.org/wiki/images/thumb/8/80/Sos_response.png/800px-Sos_response.png; 25.06.2015)

In addition to repair machineries that act on the protein level, general DNA damage repair pathways play an important role in repairing stress-related damages (Sargentini and Smith 1986, Voloshin et al. 2003, Nagashima et al. 2006, Cheng et al. 2012, Odsbu and Skarstad 2014). Organisms have developed a variety of different DNA repair pathways, which can be classified as single-strand repair, double-strand repair and others (e.g. base-excision repair, cross-links). In prokaryotes, the global SOS response system is characterized by the interplay of coprotease RecA and SOS response repressor LexA. Presence of single-strand DNA leads to activation of RecA and cleavage of the LexA protein derepressing transcription of SOS response genes as shown in Fig. 2 (Janion 2008). Studies on UV stress in *E. coli* have identified a large number of SOS box containing genes have been identified including *recF*, *recO*, *recR*, *recN* (Janion 2008) and further *recB*, *recC*, *recJ* and *radA* (Sargentini and Smith 1986). For acclimatization of species to stressful environmental conditions, both heat shock pathways for damaged protein repair and DNA repair pathways play an essential role.

To investigate the details of molecular stress responses in order to determine how species react to external stress due to environmental changes, it is important to also take the level of species interactions into account. In general, species live in interaction with other species, influencing each other on various levels. One of the most direct interactions between different species is symbiosis describing different forms of biological interactions such as mutualism, commensalism and parasitism. As one type of symbiosis, mutualism represents a beneficial association between species, which in some cases can be so close that the single mutualistic symbionts are not able to survive on their own anymore. Therefore, such a tight interaction as mutualism is particularly interesting to characterize molecular stress responses among interacting species. Mutualistic interactions are widespread in many groups of organisms in all kinds of habitats. Examples of well-studied mutualistic symbioses are coral-dinoflagellate symbioses in marine ecosystems and plant- or fungal-bacterial symbioses in terrestrial habitats (McCowen et al. 1986, Timmusk and

Wagner 1999, Yahr et al. 2004, Antonyuk and Evseeva 2006, Albright et al. 2010, Campo et al. 2013). It has been shown that both symbiosis partners can communicate and strongly influence each other at the molecular level (Timmusk and Wagner 1999, Antonyuk and Evseeva 2006). For the plant-bacterial symbiosis of wheat and the rhizobacterium *Azospirillum brasilense* it has been shown that increased gene expression of wheat lectin can upregulate metabolic processes in its bacterial symbiont (Antonyuk and Evseeva 2006). For *Arabidopsis thaliana*, the rhizobacterial symbiont *Paenibacillus polymyxa* has been shown to be responsible for increasing gene expression in the plant in reaction to drought stress (Timmusk and Wagner 1999).

Lichen symbioses are important components of a variety of terrestrial ecosystems. Lichenization is a successful nutritional strategy, which is characterized by the mutualistic association of a filamentous fungus (mycobiont) with one or several photosynthetically active organisms (photobiont), which can be green algae or cyanobacteria (Lutzoni and Miadlikowska 2009, Honegger 2012) (Fig. 3). About 20% of all fungi (17.000-20.000 species) are lichen-forming, nearly all within the phylum of Ascomycota. In contrast, only about 120 cyanobacterial and green algal species are known as photobionts in lichen symbioses (Honegger 2012). Therefore, many mycobionts share the same photobiont. Some of the most common photobionts in lichen-symbioses are green algae of the genera *Trebouxia* and *Trentepohlia* and the cyanobacterial genus *Nostoc* (Lutzoni and Miadlikowska 2009). Lichens are abundant in various ecosystems across the world reaching from arctic and antarctic tundra over alpine to steppe and desert ecosystems. More than 12% of all terrestrial ecosystems are lichen-dominated (Honegger 2012). Lichens can be very sensitive to changes in their environment such as air pollution (Hawksworth 1970, Nimis 2002). Using the bioclimatic envelope approach (statistical correlation of species distributions and environmental variables to define species tolerance), studies on British epiphytic lichens have predicted major future range shifts for lichen-forming fungi as response to climate change (Ellis et al. 2007a, Ellis et al. 2007b).

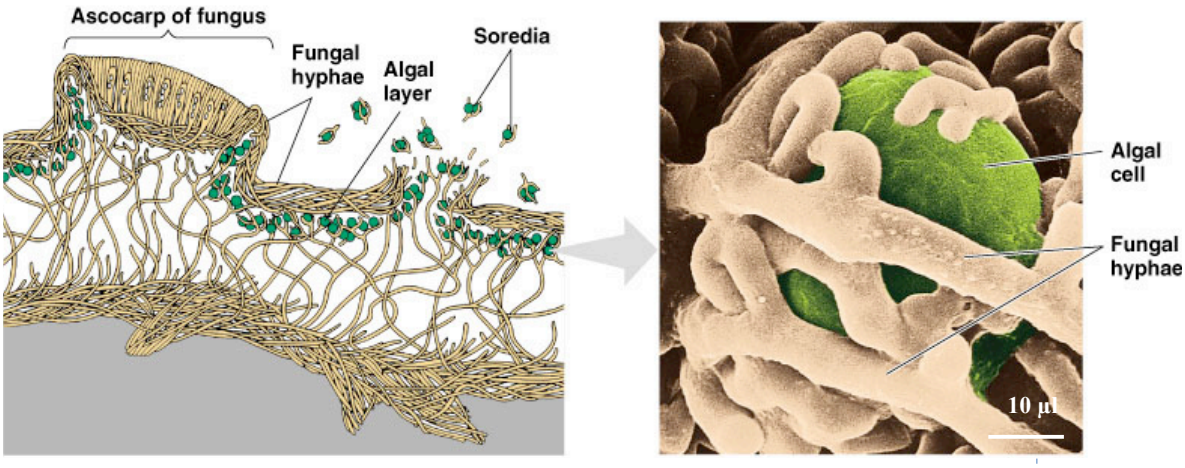

Figure 3: Lichen symbiosis. Left: schematic cross-section showing algal layer within fungal tissue. Right: false color electromicrograph of algal cell within fungal hyphae (Copyright © Pearson Education, Inc., publishing as Benjamin Cummings; http://biology4isc.weebly.com/4-kingdom-fungi.html (04.06.15))

However, it basically remains unknown how lichen populations react to large-scale climatic changes such as global warming. Choosing or switching to a specific photobiont strain better able to tolerate extreme environmental conditions than another strain, can be one strategy of coping with a changing environment (Blaha et al. 2006, Fernandez-Mendoza et al. 2011, Printzen et al. 2012). Genetic diversity and photobiont transmission mode has been investigated in many lichen symbioses either containing green-algal photobionts (Yahr et al. 2004, Piercey-Normore 2006, Yahr et al. 2006, Werth and Sork 2010, Fernandez-Mendoza et al. 2011, Grande et al. 2012, Perez-Ortega et al. 2012, Werth 2012, Widmer et al. 2012, Campo et al. 2013) or cyanobacteria (Myllys et al. 2007, Lücking et al. 2009, Fedrowitz et al. 2012). Many studies have shown that mycobionts can be highly selective for specific *Nostoc* strains as photobionts (Myllys et al. 2007, Fedrowitz et al. 2012, O'Brien et al. 2013).

Another strategy through which lichens might be able to tolerate climatic changes is flexibility in gene expression allowing the organisms to cope with environmental extremes and stress. There have been several studies on gene expression under environmental stress conditions in non-lichenized fungi (Gasch 2007) but only few recent studies have investigated gene expression in lichen-forming fungi and their photobionts. One study found differential expression of heterocyst-specific and other genes in a *Nostoc* photobiont between marginal and central parts of lichen thalli, but this study did not explicitly aim to look at stress responses (Chua et al. 2012). Another study found differential gene expression of the fungal gene *lec1* but did not consider stressful environmental conditions (Miao et al. 2012). Other studies determined differential gene expression in the context of symbiont recognition in early developmental stages of lichens (Joneson et al. 2011, Athukorala and Piercey-Normore 2015). In the desert lichen *Endocarpon pusillum*, expression of fungal genes involved in osmoregulation, metabolism and protein repair has been investigated in response to drought stress (Wang et al. 2015). However, no studies have yet been performed in order to identify and characterize gene expression of ESR genes in both lichen-forming fungi and their cyanobacterial symbionts under temperature stress.

In this study, we have investigated the temperature-stress response of different populations of the lichen-forming fungus *Peltigera membranacea* and its cyanobacterial symbiont *Nostoc* in Iceland by characterizing expression patterns of ten selected ESR genes in the mycobiont and nine in the photobiont. The membranaceous dog lichen *P. membranacea* is a terricolous lichen, which is distributed in North America, Europe, Asia and Africa (Martínez et al. 2003) (Fig. 4). *Peltigera membranacea* belongs to the *P. canina* species complex and is closely related to *P. degenii* (Miadlikowska and Lutzoni 2004). Due to its high abundance and its symbiosis with nitrogen-fixing *Nostoc* cyanobacteria, this species is one of the key players in carbon and nitrogen cycling in terrestrial ecosystems at northern latitudes (Miadlikowska and Lutzoni 2004). Determining the impact of increasing temperatures on the fungus and its photobiont under global warming is therefore an important research topic.

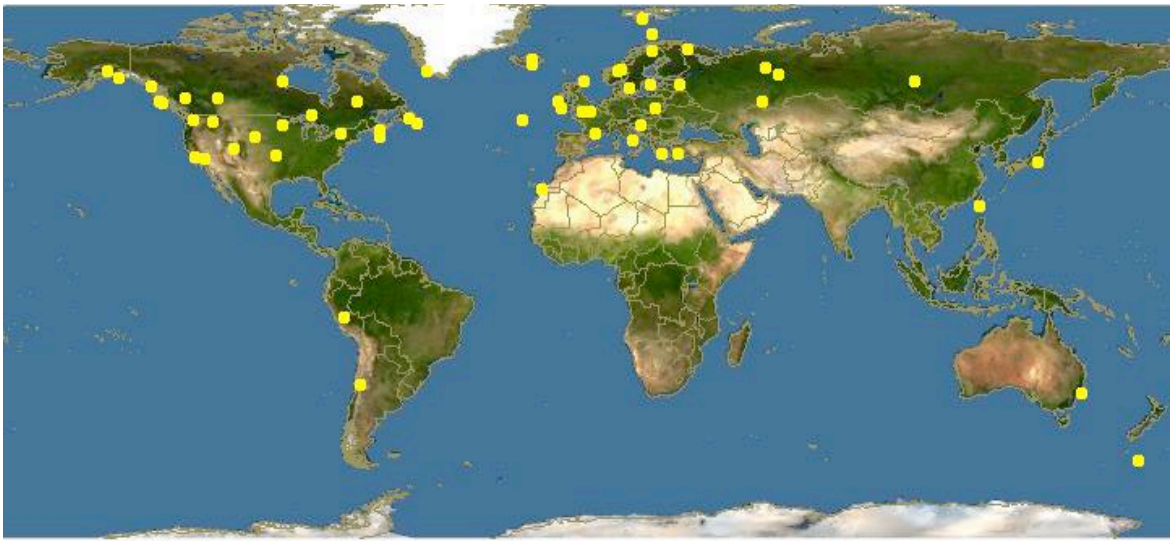

Figure 4: Worldwide distribution of the membranaceous dog lichen (*Peltigera membranacea*), http://www.discoverlife.org/mp/20m?kind=Peltigera+membranacea&flags=glean: (08.06.2015)

Gene expression of two fungal lectin genes (*lec1* and *lec2*), which are thought to play a role in the symbiosis, was also investigated in the experimental setting. The impact of fungal lectins on algal photobionts (e.g. through induction of photobiont chemotropism) has already been described for the lichens *Endocarpon pusillum*, *Xanthoria parietina*, *Evernia prunastri* and *Peltigera canina* (Molina and Vicente 2000, Legaz et al. 2004, Diaz et al. 2011, Wang et al. 2014). Expression of the *lec1* and *lec2* genes has recently been studied with regard to their role in the symbiosis between *P. membranacea* and its photobiont *Nostoc* sp. (Manoharan et al. 2012, Miao et al. 2012). Whereas *lec2* was found to be a highly polymorphic gene in *P. membranacea*, *lec1* showed differential expression between different tissues of the lichen (thallus part containing the photobiont vs. photobiont-free rhizines and ascocarps) dependent on the presence or absence of the photobiont. For wheat it has been suggested that differential expression of wheat lectin can affect cellular processes in its bacterial photobiont (Antonyuk and Evseeva 2006). Nitrogen fixation and transport of ammonium out of the cell were increased in higher lectin concentrations. Furthermore, higher lectin concentrations increased the bacterial production of the phytohormon IAA (indole-3-acetic acid), which stimulates plant growth (Antonyuk and Evseeva 2006). Therefore, the expression analysis of *lec1* and *lec2* in *P. membranacea* and genes of its photobiont *Nostoc* might provide information about the interaction of these species under increasing temperatures.

In addition to the investigation of the lichen heat-stress response in general, this study also addresses the question whether Icelandic lichens from different habitats are affected differently by increasing temperatures. As described earlier, populations of a species can differ in their response to environmental changes dependent on the natural variability of their habitat. Therefore, lichen populations from naturally more variable habitats such as in the inland of the country might have a better ability to cope with

changes in temperature than lichens from more stable habitats such as at the coast. Further, we want to determine if there is a categorical difference in temperature response or a gradual difference that is correlated with increasing distance from the sea or elevation above sea level. To answer this question, we also addressed the role of general variation between different locations within one habitat. In the Andrésson lab at the University of Iceland, unique genomic data and data on population genetics are available for *P. membranacea* and its *Nostoc* symbiont including transcriptome data, which provide a solid foundation for this study and further studies.

To address the issues described above, the following research questions have been developed:

- Does a moderate increase in temperature (5°C to 15°C to 25°C) cause a stress response reflected in the expression level of selected genes in both symbiosis partners (mycobiont and photobiont) of the lichen *P. membranacea*?

- Do lichens collected from the inland of Iceland show different temperature response patterns in these genes than lichens from coastal environments?

2 Materials and Methods

2.1 Sampling

Sampling was performed in the beginning of June 2014. Four sea-exposed sites, RY10 (Strandakirkja), RY9 (Garður), RY7 (Grindavík) and HF2 (Kjalarnes), and four sites located further inland (UX4 (Uxahryggir), LA1 (Gjábakkaheiði), HV3 (Árnes) and LL3 (Hrauneyjar), were chosen for sampling. All eight sampling sites are located in the South-West of Iceland (Fig. 5) and they represented open vegetation without forest coverage.

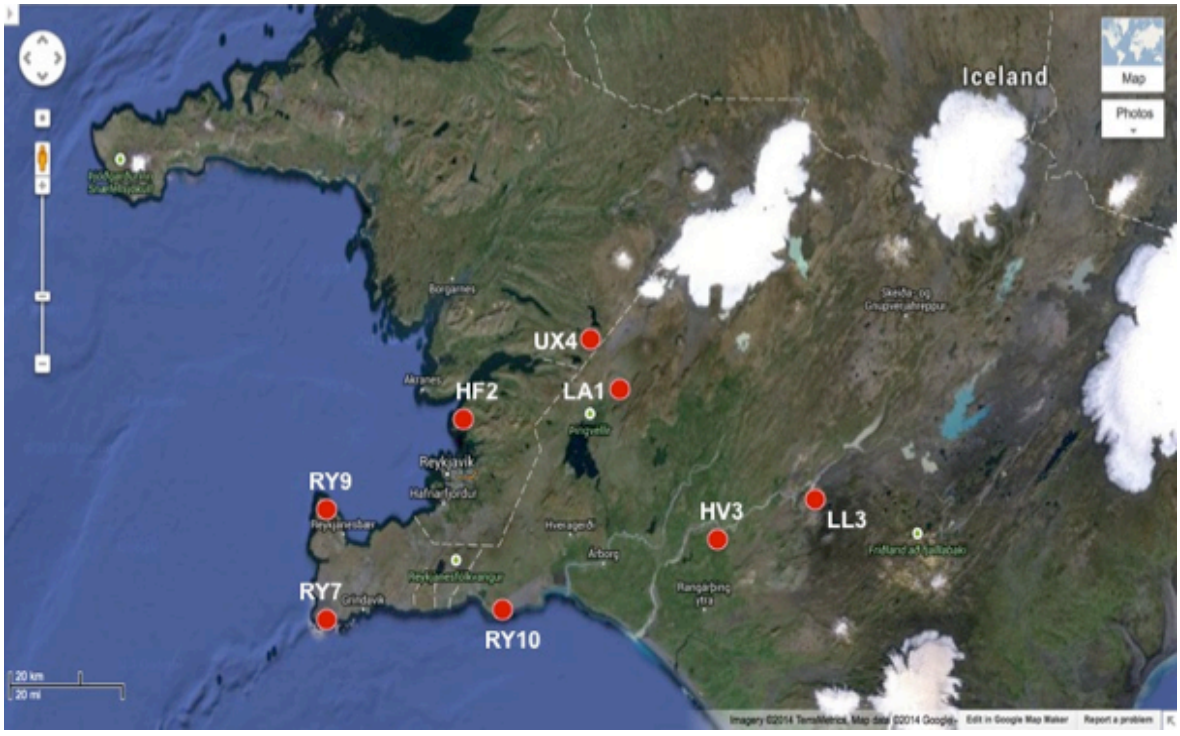

Figure 5: Map of South-West Iceland showing location of the chosen sampling sites. Located close to the sea were RY10 (Strandakirkja), RY9 (Garður), RY7 (Grindavík), and HF2 (Kjalarnes). Inland sites were UX4 (Uxahryggir), LA1 (Gjabakkaheiði), HV3 (Árnes), and LL3 (Hrauneyjar).

Table 1: Sampling site information. Table includes the exact location name, habitat, distance to the sea [km], elevation above sea level [m] and coordinates (Northing, Easting; map datum WGS84).

Site	Location name	Habitat	Sea distance [km]	Elevation [m]	Coordinates °N	°E
RY10	Strandakirkja	sea-exposed	0	10	63,835	-21,703
RY9	Garður	sea-exposed	0	7	64,080	-22,691
RY7	Grindavík	sea-exposed	0	30	63,819	-22,681
HF2	Kjalarnes	sea-exposed	0,1	31	64,240	-21,868
UX4	Uxahryggir	inland	20	415	64,438	-21,043
LA1	Gjábakkaheiði	inland	30	184	64,212	-20,860
HV3	Árnes	inland	40	68	64,036	-20,315
LL3	Hrauneyjar	inland	70	285	64,121	-19,782

At the sites RY10, RY9 and RY7, the distance to the nearest ocean shore was around 20 m and at HF2 between 100 and 150 m (Table 1). The vegetation at the sea-exposed sites was mostly characterized by bryophytes and graminoids on sandy ground (Fig. 6C). Of the inland sites, UX4 and LA1 were closest to the sea (ca. 20-30 km) and were characterized by bryophyte vegetation on a rocky ground of volcanic origin. HV3 had a distance of around 40 km to the nearest ocean shore and its vegetation was characterized by dwarf shrubs in addition to bryophytes and graminoids on rocky ground (Fig. 6B). LL3 was the most extreme inland site sampled (distance to ocean around 70 km). It was characterized by sparse vegetation, mainly bryophytes and scattered dwarf shrubs, on rocky ground.

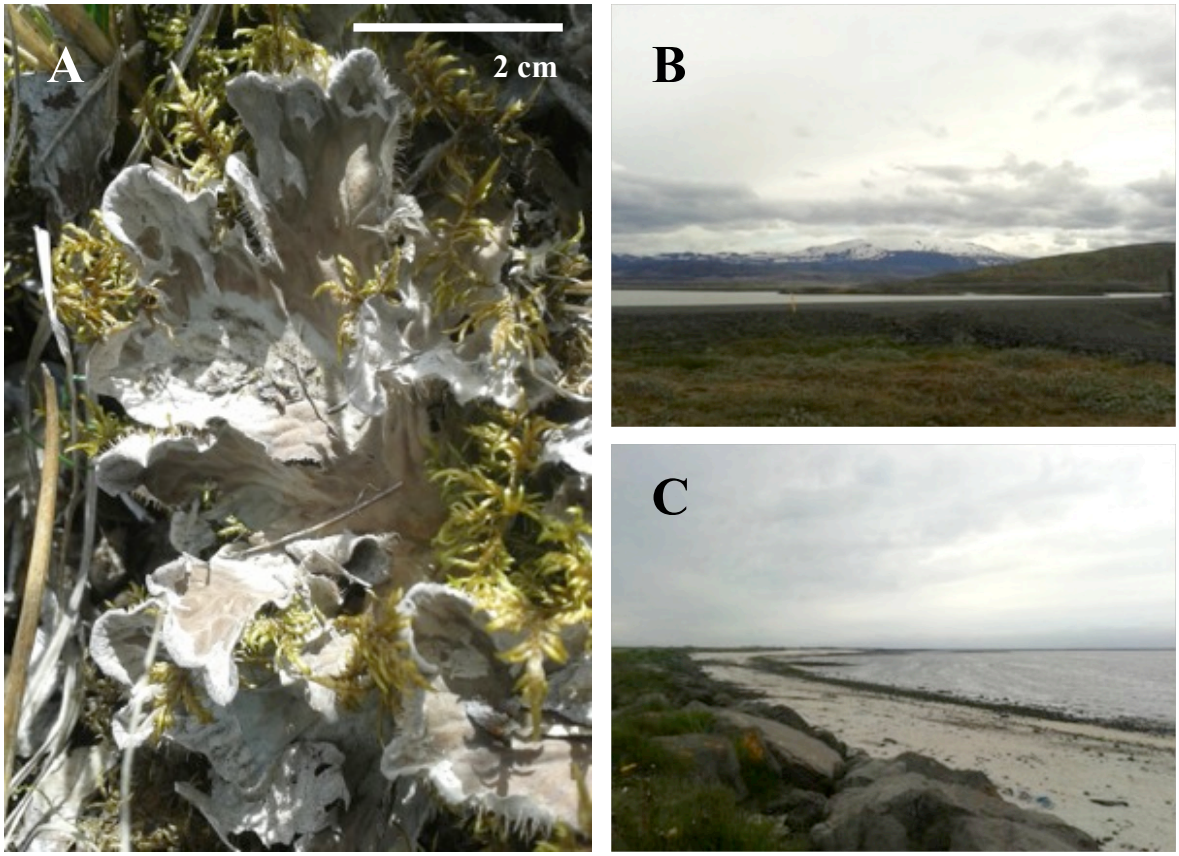

Figure 6: Organisms and habitats. A) Lichen thallus (*Peltigera membranacea*) growing among mosses; B) exemplary inland habitat (HV3), characterized by bryophytes and dwarf shrubs; C) exemplary sea-exposed habitat (RY9), vegetation characterized by bryophytes and graminoids.

Temperatures at five of the sampling sites (RY7, HF2, LA1, HV3 and LL3) were recorded in the years prior to sampling (2012 until 2014) using iButton data loggers (Maxim Integrated, San José, California, USA). The loggers were located within the vegetation on the soil so that the recorded data represent the temperatures at the level where the lichens grow. The mean temperatures in June 2013, on the same dates as sampled in June 2014, varied from 23°C at site HV3 (05.06.2013) and 7,5°C at LL3 (01.06.2013) (Fig. 7). In general, the inland sites (LA1, HV3 and LL3) showed greater variation in temperature between days than the sea-exposed sites (RY7 and HF2). An exception was site RY7 between the 20th and 30th of June, during a time when this site showed a similarly large temperature amplitude as the inland sites (Fig. 7).

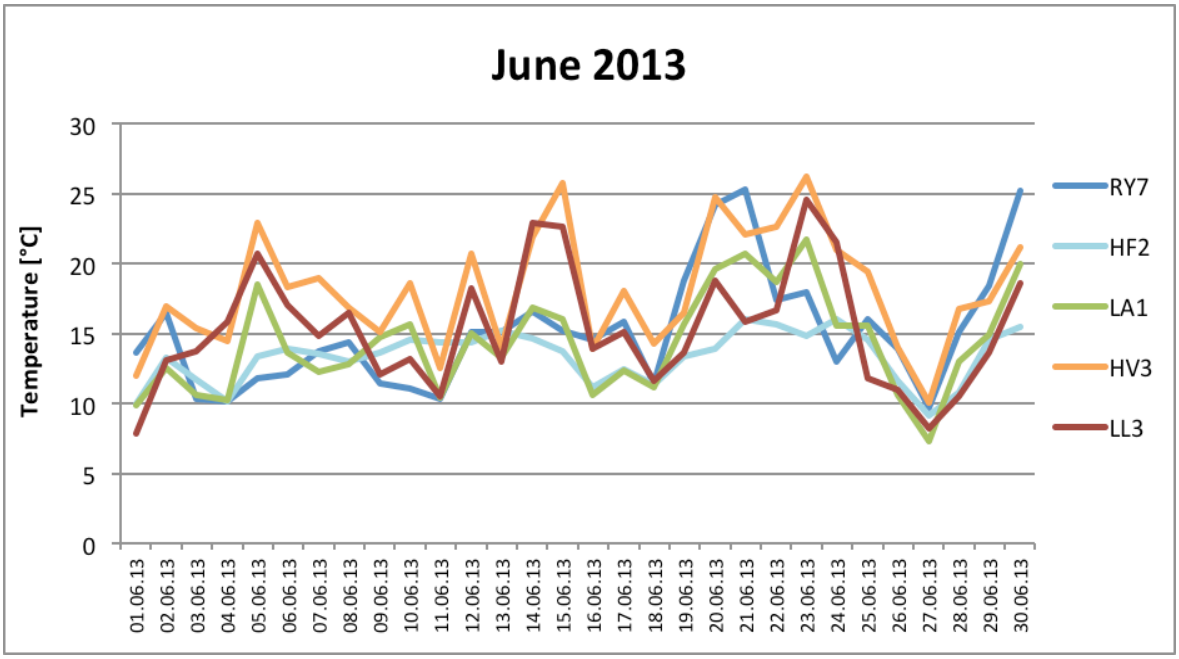

Figure 7: Daily mean temperatures at the sampling sites RY7 and HF2 (sea-exposed), as well as at LA1, HV3, and LL3 (inland) in June 2013. Mean temperature was calculated for every day based on hourly measurements (see standard deviations in supplementary table S1).

From each sampling site, five individuals of *Peltigera membranacea* (Fig. 6A) with a size of about 7 x 7 cm were randomly collected and cleaned from attached bryophytes and leaf litter. A minimum distance of ca. 1 m was kept between adjacent sampled lichen thalli in order to ensure the collection of different thalli. GPS coordinates were recorded at each sample location. All samples were stored in paper envelopes and brought to the laboratory (Askja, Háskóli Íslands), where a treatment followed to adapt the thalli to common, controlled growth conditions.

2.2 Temperature experiment

All samples, representing parts of lichen thalli (several lobes), were placed in petri dishes on filter paper and grown at 5°C under a GRO-LUX plant light (Sylvana GRO-LUX F18W/GRO $_{-T8}$, Germany) in the laboratory for 3 weeks to allow acclimation to standard conditions (light, temperature, etc.). Each of the lobes was sampled from a different lichen thallus. Samples were randomly rotated to ensure similar light exposure and were metabolically activated (by watering) and deactivated (drying out) several times during the three weeks' acclimation. After three weeks growth at 5°C, lichen tissue was sampled from each of the thalli. Then, all 40 lichen thalli were exposed to two higher temperatures, 15°C and 25°C. The samples were fully hydrated when exposed to each new temperature and kept at that temperature for 3 hours prior to tissue sampling. The temperatures were chosen based on the natural temperatures that lichens are growing under around the sampling time (Fig. 7). As shown in Fig. 7, temperatures of 25°C or even higher do occur in the natural habitat when there is wind protection or no wind and the sun is shining. For this reason, a

maximum temperature of 25°C is reasonable for investigating the effect of increased temperatures on gene expression. It represents a high temperature for a metabolically active (wet) lichen, but this temperature is regularly reached in a natural habitat. However, in the natural habitat, at 25°C, the lichens rapidly dry out and become metabolically inactive. In rainy conditions, lichens usually do not face such high temperatures in Iceland.

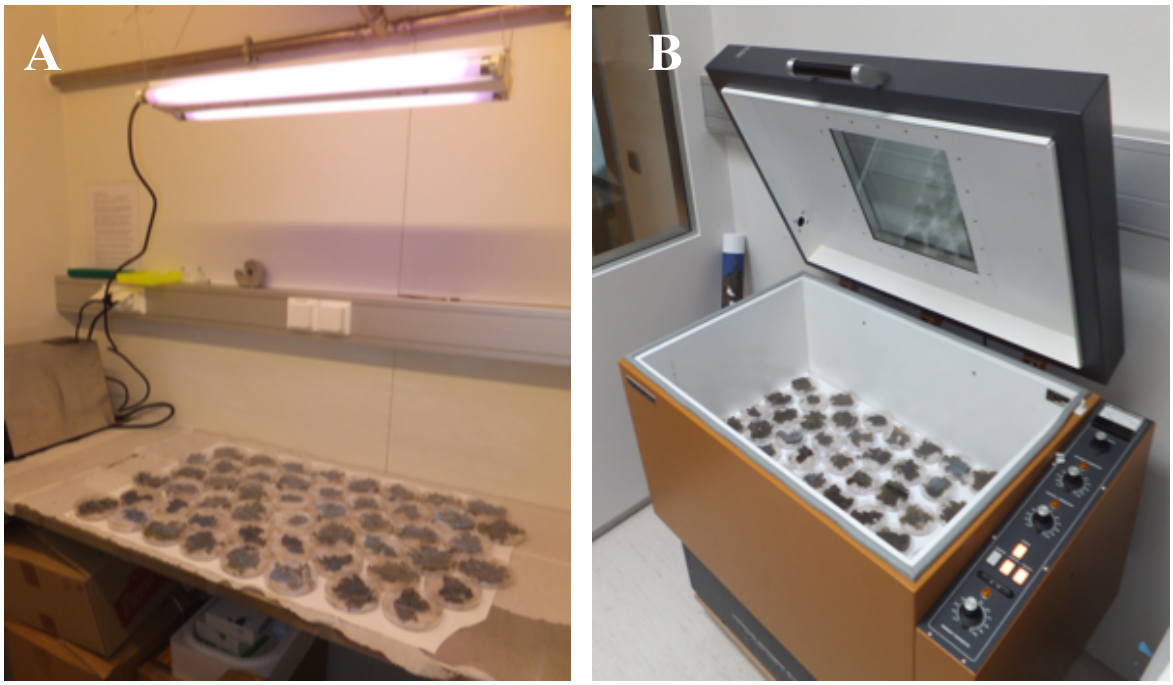

Figure 8: Setup of laboratory temperature experiment. A) Experimental setup for temperature experiment 1 (5°C treatment) in 5°C cold room, featuring controlled light conditions. Five fully hydrated lichen lobes from eight sampling sites each were set up randomly. B) Experimental setup for temperatures 2 and 3 (15°C and 25°C treatments) in controlled environment incubator, the same lichen individuals (fully hydrated, set up randomly and exposed to 15°C for 3h and 25°C for 3h under controlled light conditions prior to sampling).

The 5°C treatment was set up in a 5°C cold room (Fig. 8) whereas the 15°C and the 25°C treatments were performed in a growth chamber (Controlled Environment Incubator Shaker, New Brunswick Scientific Co.) that was set to the correct temperature beforehand and supplied with light from GRO-LUX lamps. The light intensity measured in the cold room (average 37,0 ± 4,6 Lux) was not significantly different from that measured in the growth chamber (average 28,2 ± 11,8 Lux) using an illuminance meter TES 1334A (Student´s *t*-test, p-value = 0,1132).

2.3 RNA extraction and DNase digestion

For tissue sampling, a piece of tissue (ca. 5 x 5 mm) was cut from the margin of a given lichen thallus and collected into RNAlater (Life Technologies), a solution to preserve RNA. After the second and third temperature treatment, additional pieces of tissue

were collected from the same individual, but a different lobe, resulting in three samples from each individual (5°C, 15°C and 25°C) for comparison of gene expression levels in later experiments (Fig. 9).

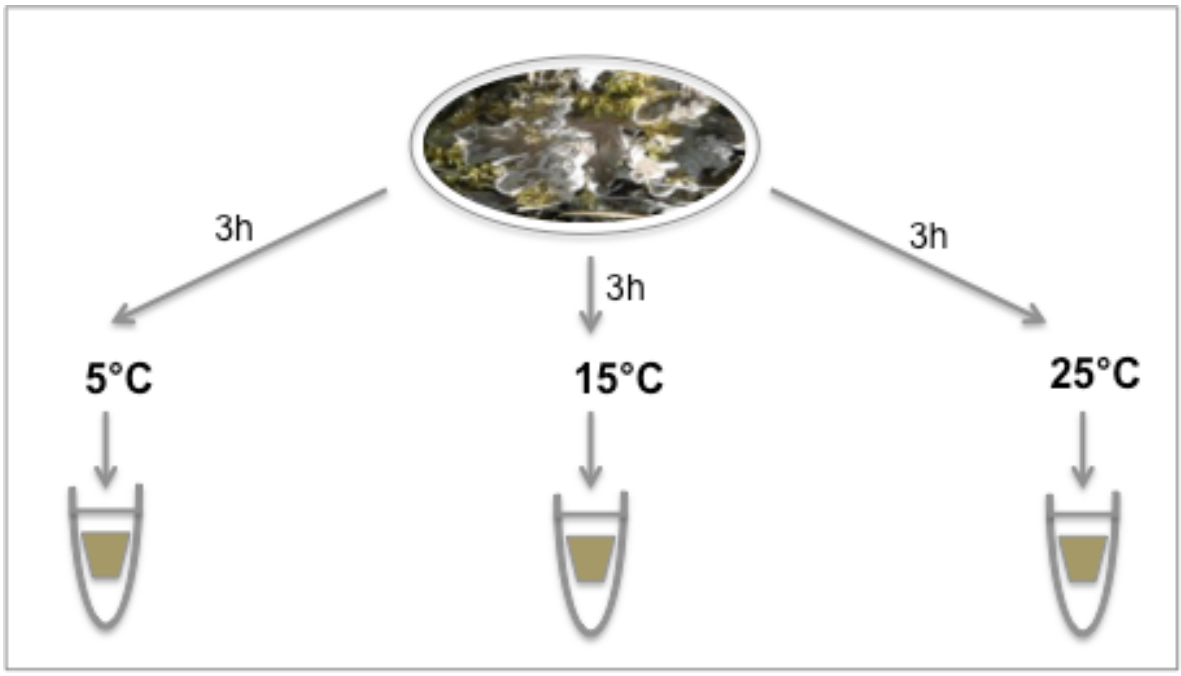

Figure 9: Experimental setup of temperature experiment. Every lichen thallus from each of the eight sampling sites was first exposed to 5°C for acclimation, then to 15°C for 3h and finally to 25°C for 3h; after every temperature treatment, pieces of marginal tissue (different lobes every time) were sampled into RNAlater for RNA extraction resulting in three temperature samples per individual.

RNA extraction was first tested using a TRI®-reagent (Sigma-Aldrich, St. Louis MO, USA) based RNA isolation protocol, but due to low and variable yields of RNA, the RNeasy Plant Mini Kit (Qiagen, Germantown MD, USA) was chosen for the extractions. RNA extractions were performed according to the manufacturer's protocol.

After elution of extracted RNA in 40 µl RNase-free H_2O and passing the eluate through the filter one more time to elute more RNA, RNA concentration and quality were checked using a NanoDrop® ND-1000 UV/Vis-Spectrophotometer (Thermo Scientific, Carlsbad CA, USA). The concentrations varied between 50 and 200 ng/µl.

In order to eliminate any remaining genomic DNA, all RNA samples were treated with DNase 1 (New England Biolabs, Ipswich MA, USA). Therefore, the extracted RNA was incubated with DNase 1 and a DNase 1 reaction buffer at 37°C for 10-15 minutes and at 75°C for 5-10 minutes in order to inactivate the enzyme (inactivation by adding EDTA was not necessary). The digested RNA samples were adjusted to a similar RNA concentration (ca. 50 ng/µl) for the following cDNA synthesis to ensure a similar RNA input in later reactions utilized for quantitative comparisons.

2.4 cDNA reverse transcription

After adjusting all samples to similar RNA concentrations, cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol (Life Technologies (Thermo Fisher Scientific), Carlsbad CA, USA). After the synthesis reaction, all cDNA samples were further diluted to a final concentration of 10 ng/µl using RNase-free H_2O. Thus, an input volume of 1 µl of cDNA sample in one RT-qPCR reaction provided a cDNA mass of 10 ng (previously determined as optimal for an efficient RT-qPCR reaction).

2.5 Quantitative Real-Time PCR (RT-qPCR)

In order to investigate whether increasing temperature had an effect on the expression levels of certain candidate genes and if this effect was dependent on the location of the sampling sites either close to the sea or inland, a gene expression study was performed using quantitative Real-Time PCR (RT-qPCR) as the main experimental approach.

2.5.1 Candidate and reference genes

The effect of increasing temperature on gene expression of fungal genes was surveyed in a previously performed RNAseq-based transcriptome study performed on *P. membranacea* (Werth & Andrésson, unpublished). Based on this study, fungal candidate genes showing expression differences at different temperatures were chosen. *Nostoc* candidate genes were chosen based on sequences deposited in GenBank as well as literature reports with a focus on DNA repair genes, heat-shock genes and chaperones, all of which were suspected to be affected by high temperatures/temperature stress (Table 2). Initially, expression of a broad set of candidate genes (20 fungal and 18 *Nostoc* genes) was tested in only two lichen individuals from one sea-exposed and one inland site in order to determine genes showing expression differences between the temperature extremes (5°C and 25°C). After confirmation of initially observed expression differences by testing individuals from another two sea-exposed and two inland sites, ten differentially expressed fungal and nine cyanobacterial candidates were determined and tested in all remaining individuals, sites and all three temperatures. The final nineteen fungal and cyanobacterial candidates varied in function from heat-shock and DNA repair genes to transcriptional regulators and symbiosis-associated genes (Table 2).

2.5.1.1 *P. membranacea*

Hhk2 and Msn2 are part of the Hog1 pathway, which is essential for inducing transcription of stress response element-containing genes under external stress (Schüller et al. 1994, Martínez-Pastor et al. 1996, Stock et al. 2000, Enjalbert et al. 2006, Gasch 2007, Schmoll 2008, Liu et al. 2013). Mot1 and Swi10 are members of the Swi/Snf2 family with Mot1 playing a role in transcriptional regulation of stress responsive TATA box-containing

genes under stress conditions and Swi10 in DNA repair (Yasuhira et al. 1999, Zanton and Pugh 2004, Dasgupta et al. 2007, Lopez-Maury et al. 2008, Sikorski and Buratowski 2009). The heat shock genes *hsp88* and *hsp98* are members of a broad heat shock response network playing an important role in preventing aggregation of denatured proteins and repair of misfolded protein due to heat stress (Vassilev et al. 1992, Plesofsky-Vig and Brambl 1998, Wang et al. 2007, Doyle and Wickner 2009). The single peptide peptidase Spp belongs to a network of membrane proteases and is thought to play a role in protein quality control by collecting damaged membrane protein aggregates for later disposal (Dalbey et al. 2012). UCRNP2_806 possibly contributes to base-excision based DNA repair. The lectin-like genes *lec1* and *lec2* were additionally chosen for expression analysis because of their believed importance in the symbiosis (Manoharan et al. 2012, Miao et al. 2012).

The *P. membranacea* glyceraldehyde-3-phosphate dehydrogenase 1 (*gpd1*) and tubulin 2 (*tub2*) genes were used as reference genes. These genes were chosen based on the literature (Manoharan et al. 2012, Miao et al. 2012) and on the previous RNAseq-based transcriptome study on *P. membranacea* (Werth & Andrésson, unpublished). Both reference genes were additionally tested for expression stability throughout the experiment, which could be confirmed (standard deviations of Ct values <10% (*gpd1* = 7,6%; *tub2* = 9,2%)). For both reference genes, Tukey´s t-test revealed no significant expression differences between temperatures and sites.

2.5.1.2 *Nostoc*

The final *Nostoc* candidate genes *hsp90* and *dnaJ* as well as heat shock chaperone *groEL* and the transcription repressor *hrcA* are involved in the cyanobacterial heat stress responses (Langer et al. 1992, Wiech et al. 1992, Hartl 1996, Mendoza et al. 1996, Goloubinoff et al. 1997, King-Chuen and Wai 1998, Young 2001, Hossain and Nakamoto 2003, Melkani et al. 2005, Rajaram and Apte 2010, Reddy et al. 2011, Wallenius et al. 2011). The *Nostoc* genes *radA*, *recF*, *recN* and *recO* are involved in the cyanobacterial SOS DNA damage repair (Sargentini and Smith 1986, Rostas et al. 1987, Odsbu and Skarstad 2014) and the candidate *Npun_F4482* might play a role base-excision DNA repair.

For the cyanobacterial photobiont *Nostoc*, the RNase P subunit B (*rnpB*) and preprotein translocase subunit A (*secA*) were chosen as reference genes as they had been validated in a previous study of *Nostoc* sp. (Pinto et al. 2012). Expression stability could be confirmed for both reference genes (standard deviation: *rnpB* = 13,9%; *secA* = 8,7%). Expression of both cyanobacterial reference genes was not significantly different between temperatures and sites.

Table 2: Reference and candidate genes for *P. membranacea* (*F)* and *Nostoc (C)*; includes gene names, functional descriptions, forward and reverse primer sequences and primer efficiencies of the two fungal and two bacterial reference genes (lines 1-4), the selected fungal candidate genes (lines 5-14) and the selected bacterial candidate genes (lines 15-23).

Gene Name		Gene Function	Forward Primer	Reverse Primer	Efficiency [%]
gpd1	***F***	Glyceraldehyde 3-phosphate dehydrogenase	CGCCGTGTTCTTGATCTCCTA	CGCCCCTCCATTCGTCATT	77,2 ± 0,04
tub2	***F***	Beta-tubulin_661	CTGACACCACAGATTAGCCCTAG	CGCCAGCACCATCTAGTCC	79,0 ± 0,05
rnpB	***C***	RNase P subunit B	GTGAGGATAGTGCCACAGAAA	CGCCTCTAGCGGCTCT	69,1 ± 0,06
secA	***C***	Preprotein translocase subunit, ATPase	ATTACATCGTCCGCAATGGGG	GCTAGAGTTTGAGTTTCTGGCTG	81,7 ± 0,05
lec1	***F***	*P. membranacea* isolate XBB013 galectin-like protein (lec-1) gene	ATCCTACGGCTTGAATAAGGGC	CGACGGATACAGTGGGAGGT	82,0 ± 0,04
lec2	***F***	*P. membranacea* isolate XBB13-9 lectin-2 gene	CTCCAACATACACACCTTCACCA	GCGTCATAGGGCAGATTCACT	78,4 ± 0,02
msn2	***F***	Zinc Finger Protein Msn2 (C2H2 type), stress-response activation	CATATCTCATCGTGTCAAACCGC	GGATGGGTGAATCGCCTC	80,0 ± 0,04
hsp88	***F***	Putative Heat-shock-protein 88	CAGACACGGAAGATAAGAAGAACGA	TGTAGCATCGTCGCCTTCG	75,1 ± 0,04
hsp98	***F***	Heat shock protein Hsp98/Hsp104/ClpA, putative [*A. flavus* NRRL3357]	GCTACTCCCCCGCATACG	CACCACAATCCTTCCATCGTCT	79,2 ± 0,03
mot1	***F***	TBP associated factor (Mot1) [*A. oryzae* RIB40]	GGACACCGTGATTTTCGTGG	GAAGAGGTCAAGGATTTGGTCTG	78,0 ± 0,06
swi10	***F***	Putative mate-type switch/DNA repair protein swi10 protein	CTCCAGCAGCAGCATCTGT	CGAATCAGAGCGAGAATTGGGTT	83,5 ± 0,03
spp	***F***	Putative signal peptide peptidase [*A. ruber* CBS 135680]	CGTCAAAGCAGAATACATCACTG	TCCATTACTGCCAGGGTTGTAA	80,2 ± 0,06
hhk2	***F***	Putative histidine kinase HHK2p [*F. verticillioides*]	ATGTTGTTTCCTTGGACATGGC	GATGTGAGCCTCCGTGGTA	83,2 ± 0,05
UCRNP2_806	***F***	Putative -gpd family base excision DNA repair protein [*N. parvum*]	CGCCACTTTCTTCCAACAAC	CGATCTTTTCCTCCAAGTCAAC	81,7 ± 0,04
groEL	***C***	Chaperonin GroEL	TGGTACAACTCTGGCTCACC	ACAGCACCATTCTGACCGG	79,2 ± 0,05
radA	***C***	DNA repair protein RadA	TTTAGGCAACCGTGACGATCC	AACCCACCCGCAGAAGCA	71,5 ± 0,05
hsp90	***C***	Hsp90 (ATPase domain protein)	CGATGCGGGGTGTGATTGAT	GCGGAAAAGTTCTTTGAGGCG	70,8 ± 0,05
Npun_F4482	***C***	Helicase domain protein	TACCAGGATGCCCAAGAATGC	TACCACTACCAGTAGCCATTTTCAG	79,8 ± 0,03
dnaJ	***C***	Chaperone DnaJ	CCTGGGTGTCTCTCGTGAC	GCGGGTTTCGGGTTCTGA	78,7 ± 0,02
hrcA	***C***	Heat-inducible transcription repressor HrcA	GGAACCAGAACCAGAGGATGC	CCCCAAAACTCCCACACTTCC	76,5 ± 0,05
recO	***C***	DNA repair protein RecO	CTGTCACAACCAGAGATAATGC	TCAAGGTAGCAGAGCGAATAG	70,8 ± 0,04
recF	***C***	DNA replication and repair protein RecF (recombinase RecF)	CTATTAACCAGACACCCGCTC	CGGGATAAATCTAGTTCGGCAAGA	77,2 ± 0,03
recN	***C***	DNA repair protein RecN	TTGGATGCCATTGATGCCG	GCTGTGATTTCTCGGCTAATGA	76,4 ± 0,05

2.5.2 RT-qPCR primer design & efficiency control

RT-qPCR primers were designed for the fungal reference and candidate genes based on transcript sequences from the RNAseq study. RNAseq data for each candidate gene were aligned against the appropriate DNA sequence in order to determine exons and introns. The primers were designed to cross the boundary of two exons in order to be unable to amplify the genomic DNA, which still contains introns. This is an important feature to ensure that the received RT-qPCR signal originates from the binding of the primers to the RNA template and not from genomic DNA contamination in the sample. All primers were designed in conserved regions.

The RT-qPCR primers for the cyanobacterial reference and candidate genes were designed based on the DNA sequences of the *Nostoc* strains N6, N232, N210a and a *Nostoc* strain sequenced from *Lobaria pulmonaria* described previously (Gagunashvili et al. 2009). The protein sequences of the selected candidate genes were used as references to annotate the coding regions in the *Nostoc* DNA and the matching sequences of the different strains were aligned for primer design. Primers were designed in conserved regions in order to cover all different *Nostoc* strains, as we expected spatial variation in *Nostoc* strains of *P. membranacea*.

The selected primer sequences were checked for self-complementarity, melting temperature (~60°C), GC-content and self 3' complementarity to the reverse primer according to the MIQE guidelines (Bustin et al. 2009). The amplicon lengths for all candidate genes varied between 90 and 220 base pairs. Primers were ordered from Microsynth, Balgach, Switzerland (HPLC purified and desalted). All primers were diluted to a working concentration of 5 µM (later 0.5 µl input in one 10 µl qPCR reaction) and first tested in normal PCR with a DNA template before using them for the qPCR. The primer efficiency of each primer pair was calculated based on a qPCR experiment using LinRegPCR version 11.0. Therefore, for each gene a linear regression analysis was performed with the raw data of a qPCR run (amplification data for each amplification cycle from 1 to 40) for all qPCR reaction wells containing one specific primer pair (all biological and technical replicates the gene was tested in). Using the slope of the resulting regression line the efficiency [%] was calculated for each gene (Bustin et al. 2009):

→ Efficiency E = $10^{-(1/\text{slope})} - 1$ (equation 1)

The primer efficiencies for all genes were around +/- 80% (Table 1).

2.5.3 Experimental procedure

For the measurement of relative expression, RT-qPCR was performed using PowerSYBRgreen® PCR Master Mix as recommended in the manufacturer´s protocol (Life Technologies (Thermo Fisher Scientific), Carlsbad, CA, USA). The RT-qPCR was conducted on 96-well optical PCR plates (4titude, UK) using a total reaction volume of 10

µl on an ABI 7500 real-time PCR System (Life Technologies (Thermo Fisher Scientific), Carlsbad, CA, USA). The reactions were run in technical duplicates (for minimizing variation due to pipetting and intra-plate differences) and with non-template controls (NTC) in each run and for each gene. The RT-qPCR was started with 2 min hold at 50°C followed by a 10 min hot start at 95°C to heat-activate the hot start polymerase. Subsequently, amplification was performed with 40 cycles of 15 s denaturation at 95°C and one minute annealing/extension at 60°C. For each gene, a dissociation step was performed at the end of the amplification phase when it was used for the first time in RT-qPCR. Therefore, the temperature was increased to 60°C-95°C in order to identify the temperature were the gene specific primers dissociate from the template. This step was performed in order to identify a single specific melting temperature for each primer pair and to check the primer specificity in the different samples.

2.5.4 RT-qPCR data analysis

For comparison, the RT-qPCR raw data from all experiments were threshold and baseline adjusted. The analysis of the RT-qPCR data was performed according to the MIQE guidelines (Bustin et al. 2009). With the Ct-values of a selected candidate gene (mean values of the two technical duplicates) and the geometric mean of Ct-values of both reference genes (*gpd1* and *tub2* for P. *membranacea* and *rnpB* and *secA* for *Nostoc*), the ΔCt-values were calculated for each individual lichen sample in each sampling site for each temperature treatment (example: delta between "Ct of target gene for individual one in site one in temperature one" and the "geometric mean of the Cts of both reference genes in individual one in site one in temperature one"):

$$\rightarrow \quad \Delta Ct = \text{mean } Ct_{\text{target gene}} - \text{geomean } Ct_{\text{both ref. genes}} \qquad \text{(equation 2)}$$

Relative expressions (relative quantity = RQ) were then calculated based on the ΔΔCt-method. For this purpose, the sample with the lowest expression level was chosen as "reference sample" and set as a standard for all comparisons (reference sample = 1):

$$\rightarrow \quad \Delta\Delta Ct = \Delta Ct - \Delta Ct_{\text{ref. sample}} \qquad \text{(equation 3)}$$

$$\rightarrow \quad \text{Relative Expression (RQ)} = 2^{-\Delta\Delta Ct} \qquad \text{(equation 4)}$$

Finally, the relative expression (RQ) values were plotted for the three different temperature treatments (5°C, 15°C and 25°C) in all eight sampling sites.

2.6 Statistical analysis

The statistical analysis of the RT-qPCR data (ΔCt values) was performed using R 3.0.2 (R Development Core team, 2013). Due to the variability that was caused by random effects that are due to the five chosen lichen thalli per sampling site, a linear mixed-effects model was chosen for the statistical analysis (Bolker et al. 2009). Since the data were in

agreement with normal distribution, a linear model could be applied. To analyze the effect of the two (fixed) factors of interest (temperature and sampling site/habitat (sea-exposed vs. inland)) a multivariate ANOVA was performed using a linear mixed effects model (R package: “nlme”) where temperature and site were defined as fixed factors whereas the lichen individual was set as random factor. If the ANOVA revealed significant results for the factor temperature, a post-hoc test (Tukey´s honest significant difference test (Tukey 1949)) was performed to determine which temperature treatments were different from one another with respect to gene expression of the candidate genes. To determine differences between sea-exposed vs. inland habitats, a mixed model using temperature and habitat (sea vs. inland) as fixed factor and individual and sites within each habitat (4 sites each) as random factor was created and analyzed using ANOVA. To differentiate if there is rather a categorical or a gradual difference in temperature response, we also analyzed the factors “sea distance” and “elevation” in a linear model for each single gene using ANOVA. Expression correlation between fungal and cyanobacterial genes was further analyzed using correlation analysis (R package: “corrgram”) As alternative approach to the single gene analysis using ANOVA, we also performed a principle component analysis (PCA) (R package: “stats”). In the PCA, the expression data for all candidate genes were transformed into principle components according to how much of the data set variability they explain (PC1 = highest variability). Using this approach, one can account for temperature and habitat differences within the gene groups contributing to the principle components that explain most of the variance in the data set. Therefore, a PCA of the gene expression data was performed in addition to the gene-by-gene analysis using ANOVA in order to compare the outcome and the quality of both statistical analyses.

3 Results

The research questions to be answered were:

(1) Does a moderate increase in temperature (5°C to 15°C to 25°C) cause a stress response reflected in the expression level of selected genes (Table 2) in both symbiosis partners (mycobiont and photobiont) of the lichen *P. membranacea*?

(2) Do lichens collected from the inland of Iceland show different temperature response patterns in these genes than lichens from coastal environments?

A multivariate ANOVA was performed using a linear mixed effects model with temperature and site as fixed factors and the lichen individual as a random factor (Table 2, Table 4). In cases when the ANOVA revealed significant results for the factor temperature, a Tukey´s honest significant difference test (Tukey´s Test) was performed to determine the effect of the three temperature treatments on the expression of the candidate genes (Table 3, Table 5). To further investigate research question 2 (habitat differences), a mixed model with temperature and habitat (sea vs. inland) as fixed factors and individuals and sites within each habitat (4 sites each) as random factors was used for ANOVA. To differentiate categorical from gradual differences in temperature response, the factors "gradually increasing distance from the sea (sea distance)" and "elevation above sea level (elevation)" were analyzed in addition to categorical habitats (sea-exposed vs. inland) using a linear model performed for each gene separately (ANOVA). Expression correlations among fungal and cyanobacterial genes were further examined using correlation analysis. As an alternative approach to the single gene analysis, we performed a principle components analysis (PCA) determining temperature responses and habitat differences of groups of genes based on their contribution to the total variation in gene expression.

3.1 Single gene expression responses (ANOVA)

3.1.1 *P. membranacea*

Out of 20 tested candidate genes for *P. membranacea,* the heat shock genes *hsp88* and *hsp98*, the DNA repair gene *UCRNP2_806*, the zinc finger protein gene *msn2*, the TBP associated factor gene *mot1*, the histidine kinase gene *hhk2*, the signal peptide peptidase gene *spp,* the mating-type switching protein gene *swi10* and lectin gene *lec2* (nine genes in total; see Table 2) showed significant expression differences at increased temperatures relative to the reference genes *gpd1* and *tub2*. The reference gene expression remained stable among temperatures and sites.

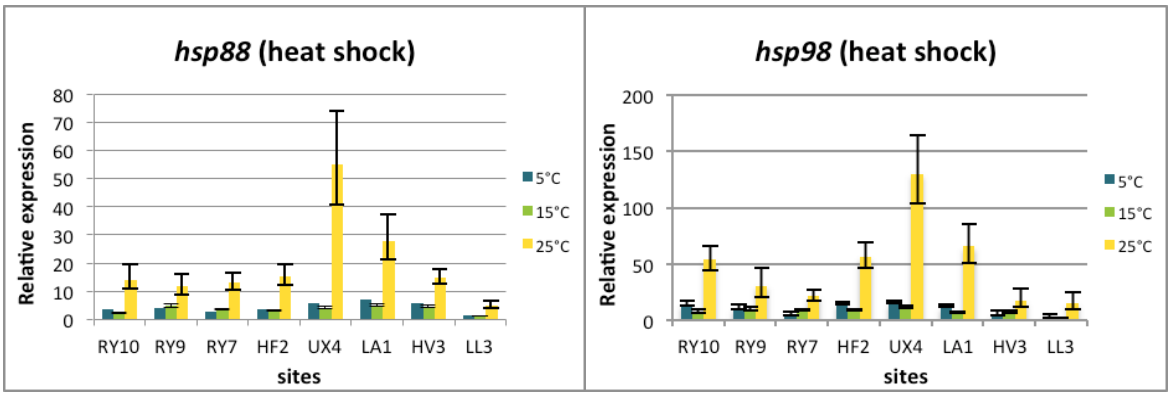

Figure 10: Relative gene expression (RQ) of fungal heat shock genes *hsp88* and *hsp98,* plotted for all eight sampling sites at 5°C, 15°C and 25°C; the sample with lowest expression over all sites and temperatures was set as reference (RQ = 1). Sampling sites were arranged from sea-exposed (RY10) to inland (LL3).

ANOVA revealed a significant effect of increasing temperature on gene expression of the heat shock genes *hsp88* and *hsp98* (Fig. 10, p-values see Table 3). Tukey´s test revealed that the expression of both genes was significantly upregulated at 25°C compared to 5°C and 15°C (p-values see Table 4). For both genes, expression differed significantly between sites and there was a site-related difference in temperature response (interaction) (Table 3). The difference appeared to be correlated with sea distance rather than with elevation or categorical habitats (sea vs. inland) (Table 3).

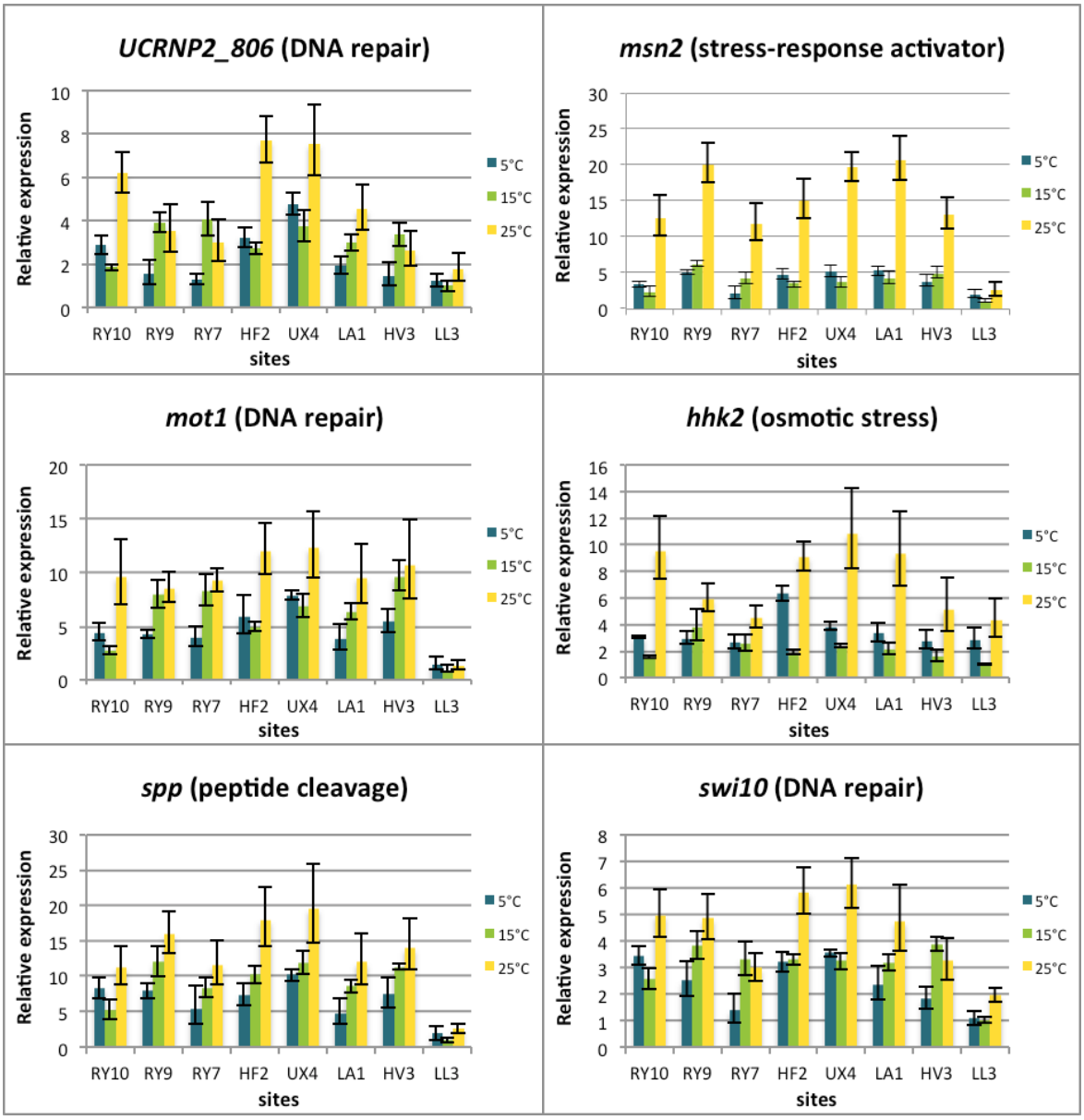

Figure 11: Relative gene expression (RQ) of fungal genes *UCRNP2_806*, *msn2*, *mot1*, *hhk2*, *spp* and *swi10* plotted for all sampling sites (arranges from sea-exposed to inland) at 5°C, 15°C and 25°C; the sample with the lowest expression was set as reference (RQ = 1). See also legend of Fig. 10.

For the six fungal genes *UCRNP2_806*, *msn2*, *mot1*, *hhk2*, *spp* and *swi10* multivariate ANOVA (fixed effects: temperature, site) revealed significantly higher gene expression levels at increased temperatures as well (Fig. 11, p-values Table 3). Expression of all six genes was significantly increased at 25°C compared to 5°C and also compared to 15°C. For *UCRNP2_806, hhk2 and swi10,* increased expression already occurred at 15°C (Table 4). Furthermore, expression of all six genes differed significantly between sites and for *UCRNP2_806, mot1* and *hhk2* also the temperature response in expression was different between sites (Table 3). However, these differences were rather associated with increasing sea-distance (and for *mot1* and *spp* also with elevation) than with the categorical variable habitat (sea vs. inland) (Table 3).

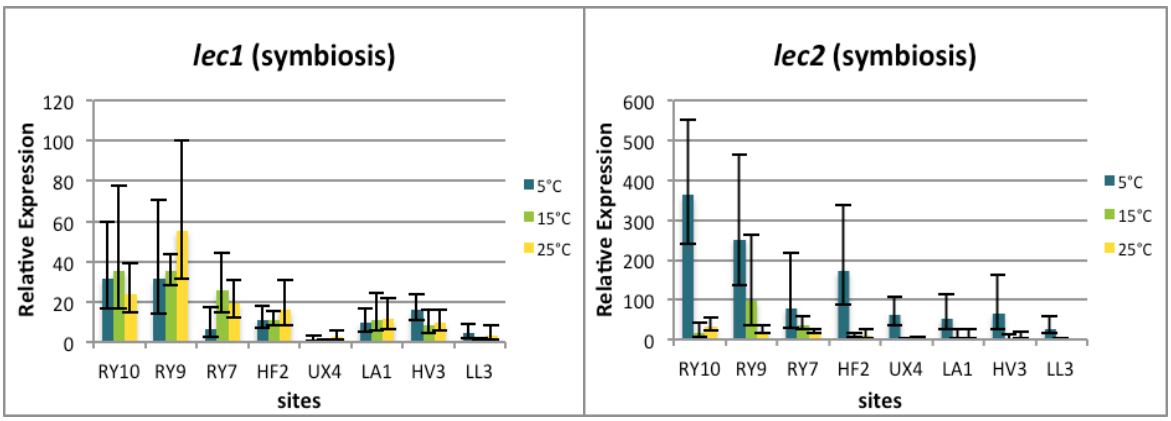

Figure 12: Relative expression (RQ) of galectin-like genes *lec1* and *lec2* for all sampling sites at 5°C, 15°C and 25°C; sample with lowest expression set as reference (RQ = 1). See also legend of Fig. 10.

Gene expression patterns of the two galectin-like genes *lec1* and *lec2,* which are thought to be important in the lichen symbiosis (Manoharan et al. 2012, Miao et al. 2012), were quite different. While expression of *lec1* was not significantly affected by temperature, *lec2* expression was significantly downregulated at 15°C and 25°C compared to 5°C. Expression of both lectins differed among sites, which was not only dependent on sea distance and elevation but also on categorical habitats (sea vs. inland) (Table 3).

Table 3: Significance (p-values) of multivariate ANOVA for fungal genes. Column 2-4: ANOVA results of linear mixed-effects model using temperature and site as fixed and lichen individual as random factor. Column 5-6: ANOVA results of linear mixed-effects model with temperature and habitat as fixed and site and individual within site as random factors. Column 7: temperature and sea-distance as linear factor (linear model). Column 8: temperature and elevation as linear factor (linear model). Significant effects on gene expression (ΔCt values) are marked with grey shading.

1 **Fungal gene**	2 **temperature**	3 **site**	4 **interaction temp x site**	5 **habitat**	6 **interaction temp x habitat**	7 **sea distance**	8 **elevation**
hsp88	<0,0001	<0,0001	0,0069	0,7411	0,1085	0,0037	0,5120
hsp98	<0,0001	<0,0001	0,0290	0,5699	0,0796	<0,0001	0,9940
UCRNP2_806	<0,0001	<0,0001	0,0037	0,6022	0,5349	<0,0001	0,6880
msn2	<0,0001	<0,0001	0,0602	0,6704	0,2155	<0,0001	0,1091
mot1	<0,0001	<0,0001	0,0261	0,6044	0,3893	<0,0001	0,0369
hhk2	<0,0001	0,0002	0,0095	0,4847	0,3800	0,0121	0,9680
spp	<0,0001	<0,0001	0,6052	0,4749	0,9782	<0,0001	0,0280
swi10	<0,0001	<0,0001	0,1218	0,4512	0,9640	<0,0001	0,1670
lec1	0,5903	<0,0001	0,9046	0,0353	0,1855	<0,0001	<0,0001
lec2	<0,0001	<0,0001	0,8560	0,0074	0,1991	<0,0001	<0,0001

Table 4: Significance (p-values) of Tukey´s honest significant difference test for the effect of the three temperature treatments (5°C vs. 15°C, 5°C vs. 25°C and 15°C vs. 25°C) on gene expression (ΔCt values) for fungal candidate genes (linear mixed-effects model: temperature and site = fixed factors, lichen individual = random factor). Significant values are marked with grey shading.

Fungal gene	5°C vs. 15°C	5°C vs. 25°C	15°C vs. 25°C
hsp88	0,1665	<0,0001	<0,0001
hsp98	0,0383	<0,0001	<0,0001
UCRNP2_806	0,0358	<0,0001	0,0011
msn2	0,6539	<0,0001	<0,0001
mot1	0,3001	<0,0001	0,0001
hhk2	<0,0001	<0,0001	<0,0001
spp	0,4230	<0,0001	0,0004
swi10	0,0263	<0,0001	0,0004
lec1	0,9559	0,7561	0,5789
lec2	<0,0001	<0,0001	0,9954

In short, expression of *hsp88* and *hsp98*, *UCRNP2_806*, *msn2*, *mot1*, *hhk2*, *spp* and *swi10* was significantly upregulated (Figs. 10 and 11) whereas *lec2* expression was downregulated with increasing temperatures. Gene expression of these nine genes as well as of *lec1* further differed significantly between sites and for *hsp88*, *hsp98*, *UCRNP2_806*, *mot1* and *hhk2* the temperature response in gene expression was also different between sites. For *lec1* and *lec2,* a categorical difference in expression between habitats (sea vs. inland) was found (Fig. 12). The gradual distance to the sea and the elevation above sea level significantly influenced expression of many more genes than the categorical difference between sea-exposed vs. inland habitats. However, neither sea distance nor elevation correlated significantly with the temperature response in gene expression (i.e. no interaction effects). Generally, the effect of temperature on gene expression was far greater than the effect of sampling sites as indicated by the consistently larger F-values for temperature in the ANOVA test statistic (see supplementary Table S4).

3.1.2 *Nostoc*

Of all 18 investigated genes for the *Nostoc* photosymbiont, expression of the DNA repair genes *radA*, *recF*, *recN* and *recO*, the heat shock chaperone genes *groEL* and *dnaJ*, heat shock gene *hsp90*, helicase-domain containing gene *Npun_F4482* and heat-inducible transcription repressor gene *hrcA* (9 genes in total; see Table 2) was significantly different at increased temperatures relative to the two reference genes *rnpB* and *secA*. Expression of rnpB and secA remained stable among temperatures and sites.

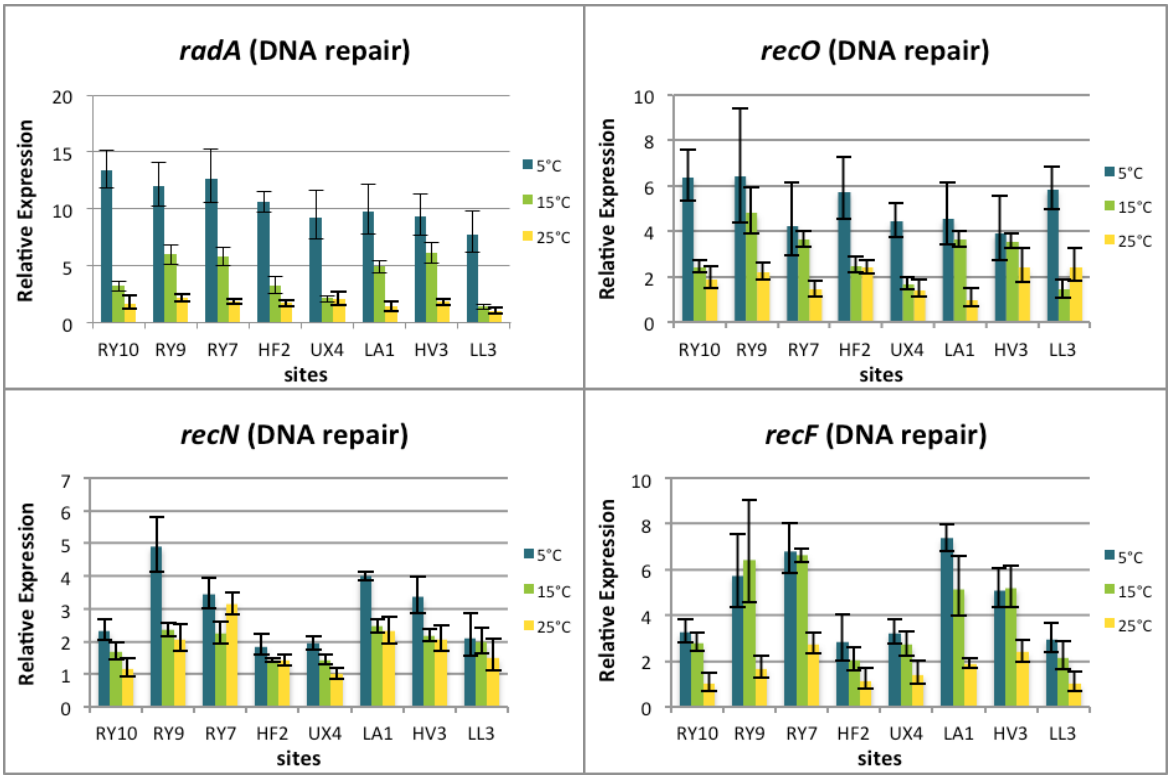

Figure 13: Relative gene expression (RQ) of cyanobacterial genes *radA*, *recO*, *recN* and *recF* for all sampling sites at 5°C, 15°C and 25°C; sample with lowest expression set as reference (RQ = 1). For further information, see legend of Fig. 10.

For the cyanobacterial DNA repair genes, expression of *recF* was significantly downregulated at 25°C compared to 5°C whereas *radA*, *recO* and *recN* expression was significantly downregulated already at 15°C compared to 5°C. Expression of *recF*, *radA* and *recO* was again significantly decreased at 25°C compared to 15°C (Table 6). For *recF*, *recN* and *radA*, gene expression also differed between sites. For *recF* and *recO*, the temperature response in gene expression differed between sites as well (interaction effect). There was no significant categorical difference between sea and inland habitats, but the expression of all four genes showed a significant correlation with increasing elevation. For *radA*, gene expression was correlated with sea distance (Table 5).

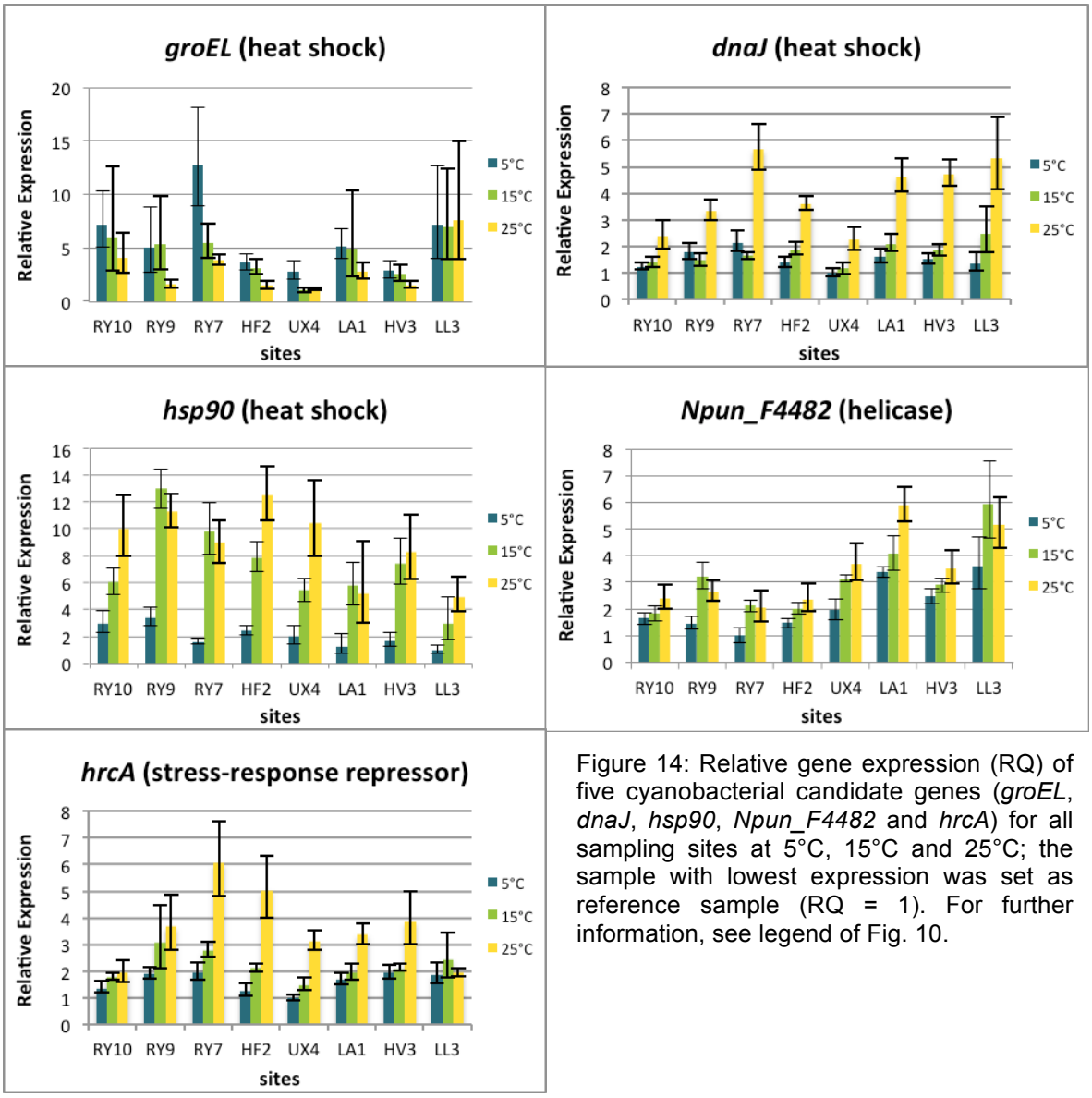

Figure 14: Relative gene expression (RQ) of five cyanobacterial candidate genes (*groEL*, *dnaJ*, *hsp90*, *Npun_F4482* and *hrcA*) for all sampling sites at 5°C, 15°C and 25°C; the sample with lowest expression was set as reference sample (RQ = 1). For further information, see legend of Fig. 10.

The expression of the chaperone gene *groEL* decreased significantly at 25°C compared to 5°C and also compared to 15°C (Table 6) and differed considerably between sampling sites. However, neither categorical habitat nor sea distance nor elevation showed significant correlation with gene expression (Table 5). The chaperone gene *dnaJ* was significantly upregulated in its expression at 25°C compared to 5°C and 15°C (Table 6). *dnaJ* expression also differed between sites, where sea distance showed only a marginally significant correlation (Table 5). Similar to the fungal heat shock genes *hsp88* and *hsp98,* the heat shock gene *hsp90* in *Nostoc* and the cyanobacterial helicase domain-containing gene *Npun_F4482* showed significantly higher expression both at 15°C and 25°C compared to 5°C (Table 6). Expression of both photobiont genes differed significantly between sites and habitat; sea distance and elevation showed significant correlations with gene expression (Table 5). The transcription repressor gene *hrcA* showed a significant expression increase at 15°C vs. 5°C and at 25°C vs. 15°C (Table 6), in addition to

expression differences between sites. Moreover, in this case there was a significant interaction between temperature response and site and a significant difference in gene expression according to elevation (Table 5).

Table 5: Significance (p-values) of multivariate ANOVA for cyanobacterial genes. Column 2-4: ANOVA results of linear mixed-effects model using temperature and site as fixed and individual as random factor. Column 5-6: ANOVA results of linear mixed-effects model with temperature and habitat as fixed and site and individual as random factors. Column 7: temperature and sea-distance (linear model). Column 8: temperature and elevation (linear model). Significant effects on gene expression (ΔCt values) are marked in grey shading.

1 **Fungal gene**	2 **temperature**	3 **site**	4 **interaction temp x site**	5 **habitat**	6 **interaction temp x habitat**	7 **sea distance**	8 **elevation**
radA	<0,0001	<0,0001	0,0254	0,1662	0,7230	<0,0001	0,0001
recO	<0,0001	0,1023	0,0380	0,1462	0,7478	0,2400	0,0074
recN	<0,0001	<0,0001	0,4531	0,8699	0,5734	0,7980	0,0032
recF	<0,0001	<0,0001	0,9828	0,9404	0,6719	0,2200	0,0356
groEL	0,0014	<0,0001	0,9073	0,4896	0,2738	0,4460	0,0738
dnaJ	<0,0001	<0,0001	0,3926	0,8768	0,0645	0,0514	0,1100
hsp90	<0,0001	0,0001	0,8684	0,0339	0,7788	<0,0001	0,0019
Npun_F4428	<0,0001	<0,0001	0,7440	0,0060	0,4919	<0,0001	<0,0001
hrcA	<0,0001	0,0001	0,0297	0,3894	0,3752	0,1075	0,0051

Table 6: Significance (p-values) of Tukey´s honest significant difference test for the effect of the three temperature treatments (5°C vs. 15°C, 5°C vs. 25°C and 15°C vs. 25°C) on gene expression (ΔCt values) for cyanobacterial candidate genes (linear mixed-effects model: temperature and site = fixed factors, individual = random factor). Significant values are marked with grey shading.

Nostoc gene	**5°C vs. 15°C**	**5°C vs. 25°C**	**15°C vs. 25°C**
radA	<0,0001	<0,0001	<0,0001
recO	<0,0001	<0,0001	0,0024
recN	<0,0001	<0,0001	0,3365
recF	0,4232	<0,0001	<0,0001
groEL	0,3238	0,0010	0,0627
dnaJ	0,1905	<0,0001	<0,0001
hsp90	<0,0001	<0,0001	0,2062
Npun_F4428	<0,0001	<0,0001	0,4940
hrcA	0,0020	<0,0001	<0,0001

In short, for the DNA repair genes *radA*, *recF*, *recN* and *recO* and the chaperone gene *groEL* the increase in temperature was associated with a significant decrease in

expression. In contrast, for the chaperone gene *dnaJ*, the heat shock gene *hsp90*, the helicase domain-containing gene *Npun-F4482* and the heat-inducible transcription-repressor gene *hrcA,* expression was significantly increased in higher temperatures (Fig. 13). Expression of all genes except *recO* differed between sites. For the genes *radA*, *recO* and *hrcA,* also the temperature response in expression differed significantly between sites (interaction). However, F-values indicated a stronger effect of temperature than site on gene expression (see supplement Table S4). For *Npun_F4482* and *hsp90,* a difference in expression between habitat categories (sea vs. inland) was found. However, elevation above sea level and sea distance showed stronger correlations with expression differences for many more genes than the categorical difference between sea-exposed vs. inland habitats (Table 4).

3.2 Single gene correlations

To investigate correlations among and between fungal and cyanobacterial candidate genes, a correlation analysis was performed with the RT-qPCR gene expression data (ΔCt values, normalized to reference gene expression) in order to determine which genes showed similar expression patterns with response to the factors temperature and sampling site. Since the same RNA isolates were used to test both fungal and cyanobacterial candidate genes, it was possible to determine correlations both among and between fungal and cyanobacterial genes.

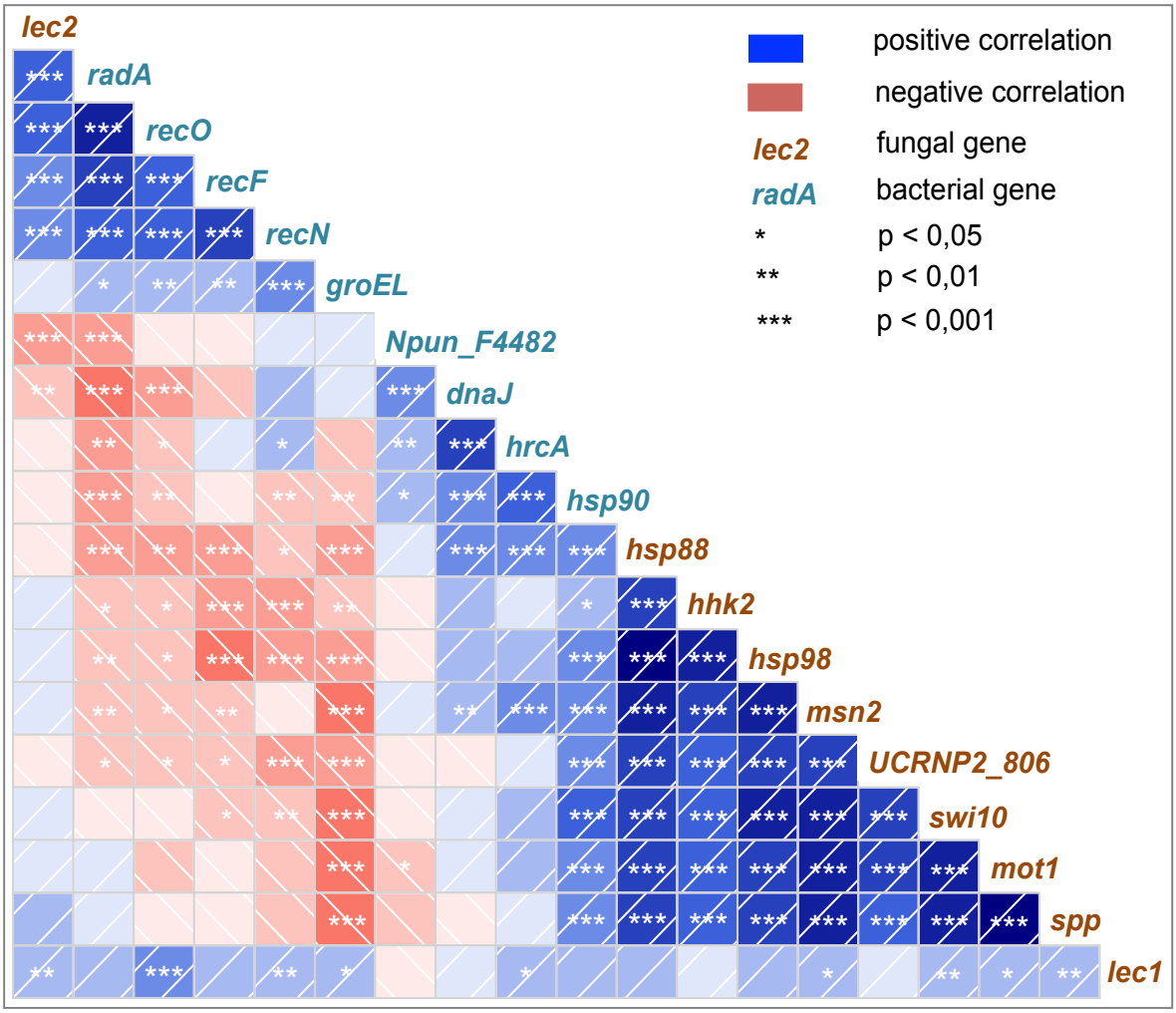

Figure 15: Correlation analysis for fungal and cyanobacterial candidate genes; shown are positive (blue) or negative (red) correlations in gene expression patterns for all mycobiont and photobiont (*Nostoc*) genes, including interspecific correlations; genes were grouped according to correlation; colour shading scheme is based on correlation coefficients (see supplement table S9); stars represent significant p-values.

Correlation analysis revealed that expression of the fungal lectin gene *lec2* was positively correlated to the expression of the cyanobacterial DNA repair genes *radA*, *recO*, *recF* and *recN* (Fig. 15). The majority of the other fungal candidate genes were grouped indicating strong expression correlations. The cyanobacterial DNA repair genes mentioned before, and especially the *Nostoc* heat shock gene *groEL,* were negatively correlated in gene expression with cyanobacterial heat shock genes and with most of the fungal candidate genes involved in DNA repair and heat stress response. In contrast, expression of the *Nostoc* stress-induced transcriptional repressor *hrcA* was positively correlated with fungal and cyanobacterial heat shock genes (*hsp90*, *hsp88*) and the fungal stress-induced transcriptional activator *msn2*. Generally, the cyanobacterial heat shock genes (*dnaJ*, *hsp90*) were positively correlated in expression pattern with the fungal heat shock genes (*hsp88*, *hsp98*) and fungal *msn2*. The second fungal lectin gene *lec1* was correlated in expression with *lec2* but was found separately from other gene groups and showed only a small number of strong correlations with other genes.

3.3 Principle component analysis (PCA)

As an alternative approach to the single gene analysis using ANOVA, principle component analysis (PCA) was performed in order to identify the main contributors to the overall data variance by principle components. This allowed the determination of the contribution of the generated gene groups to the variation within the data set (shown for the first seven PCs in Fig. 16). The results revealed that the principle components PC1 to PC3 explained the majority of the variation in the data set (~80%). Therefore, further analysis of the contributions of temperature, sites, and further categorical habitat, sea distance and elevation focused on PC1-3.

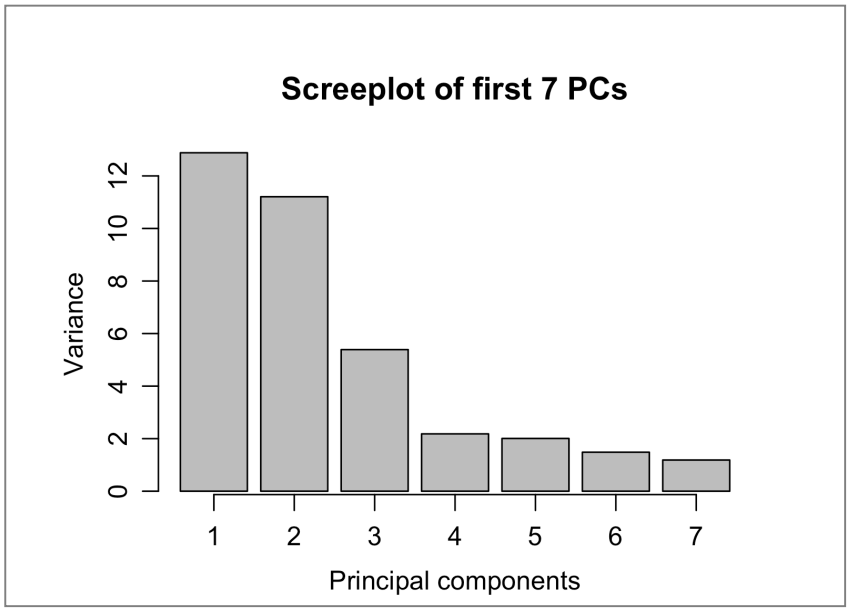

Figure 16: Scree plot showing contribution of principle components PC1 to PC7 (x-axis) to the overall data variation (eigenvector, y-axis).

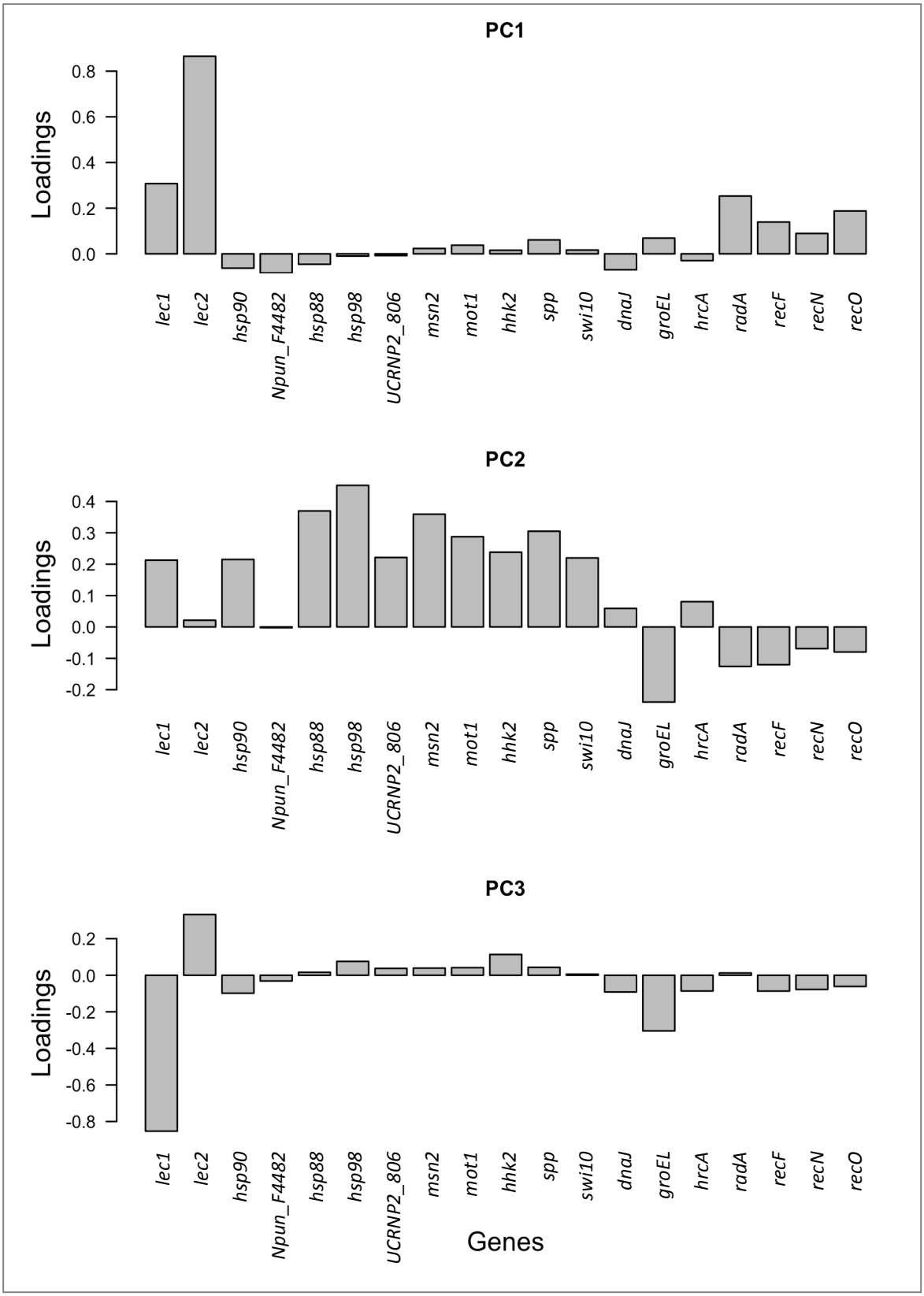

Figure 17: Axis loadings of the first three axes, PC1-3. The figure shows to what extent individual genes contribute to each principle component. Positive or negative loadings represent the direction of the contribution relative to the PC vector.

The highest contribution to PC1 was by the fungal lectin gene *lec2* (~85%) and further by *lec1* (~30%) as well as the cyanobacterial DNA repair genes *radA*, *recF*, *recN* and *recO* (~10-30%) (Fig. 17). PC2 was mainly composed of the majority of the other fungal candidate genes with smaller contributions by the fungal gene *lec1*, cyanobacterial *hsp90* and others. For PC3, the fungal gene *lec1* was the major contributor, followed by smaller contributions of *lec2* and *groEL*. However, the contribution of *lec1* and *lec2* were opposite in direction.

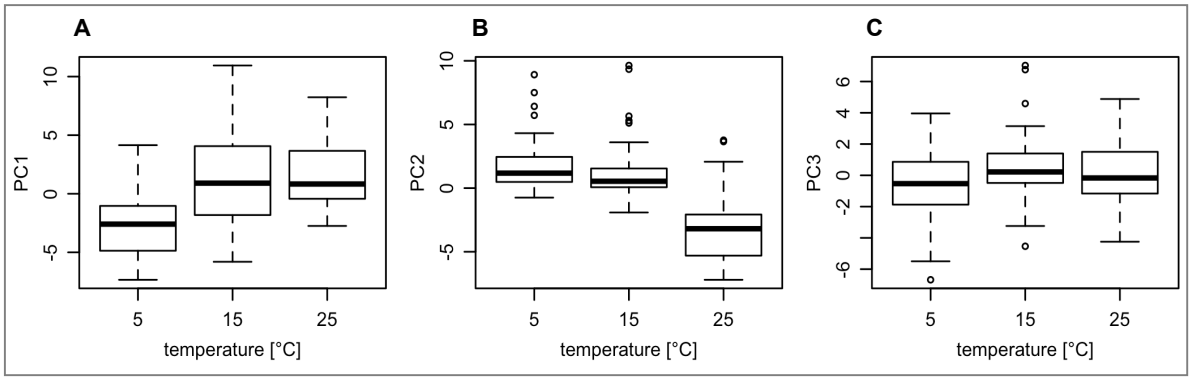

Figure: 18: The effect of temperature (15°C and 25°C compared to 5°C) on gene expression for the first three principle components PC1-3. (A) PC1, (B) PC2 and (C) PC3; x-axis: temperature, y-axis: axis scores created by the transformation of the gene expression data for PC1-3. Boxes enclose upper and lower quartile around the median (the line separating the upper and lower half of the data) and upper and lower whiskers represent 95% of data (outliers shown as dots).

There were significant differences between temperature treatments in PC1 and PC2 but not in PC3 (Table 6). In PC1, which was composed mainly of the genes *lec2* and partly *lec1*, *radA*, *recF*, *recN* and *recO*, there was a significant decrease in expression between 5°C compared to 15°C and to 25°C (Table 7, Figs. 12, 13 and 18). In contrast, PC2 containing the major set of other fungal candidate genes (heat-stress response, DNA repair) was characterized by a significant increase in gene expression occurring at 25°C compared to 5°C and 15°C (Table 7). In PC3, mainly characterized by *lec1* contribution, there was no significant difference in gene expression between the three temperatures (Table 7).

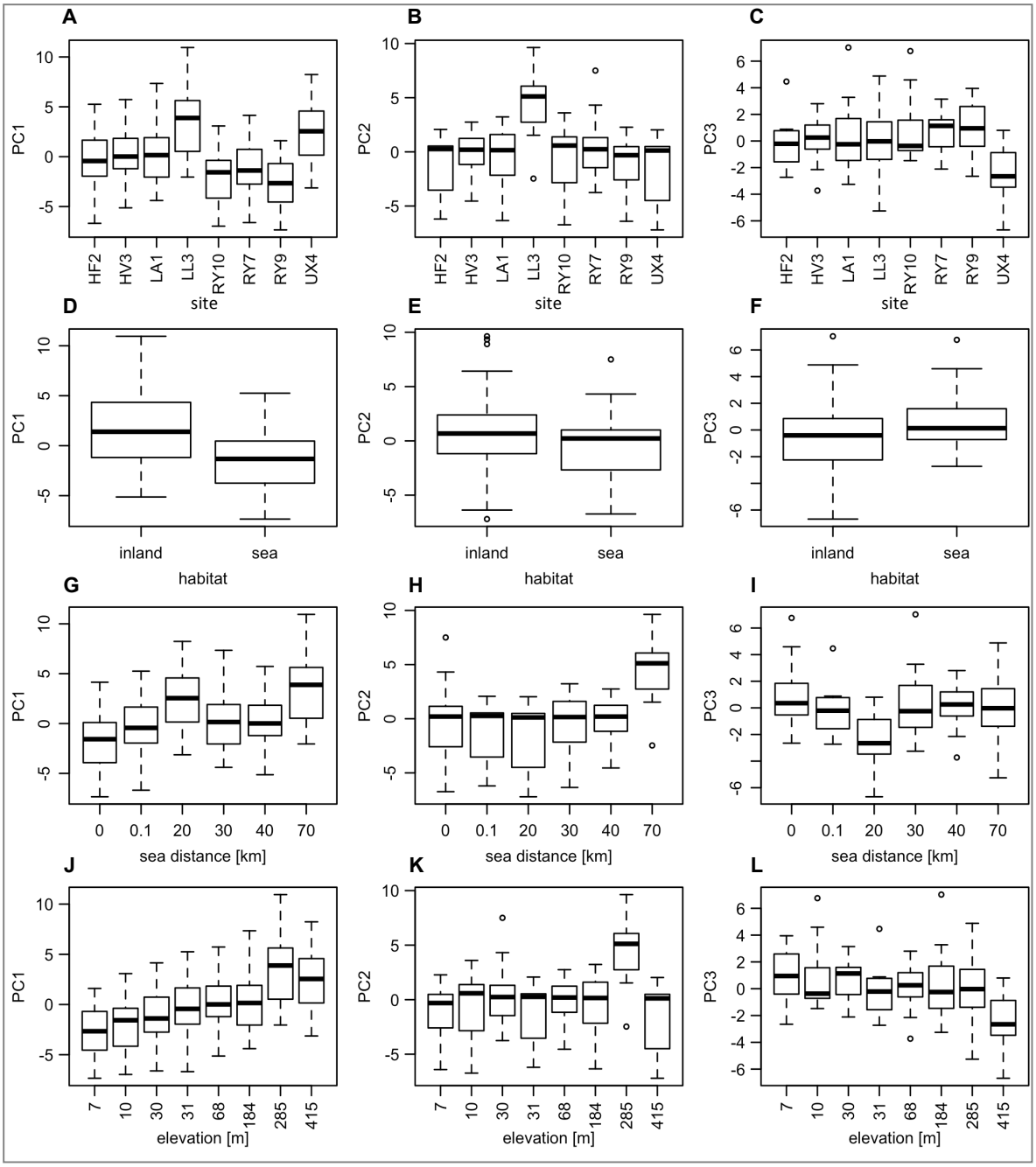

Figure 19: Differences in gene expression between the sampling sites and between habitats (sea vs. inland) as well as along sea distance and elevation gradients. (A-C) Comparison of differences in PC1, PC2 and PC3 between the sampling sites, (D-F) between categorical habitats, (G-I) with sea distance and (J-L) with elevation above sea level; y-axes: axis scores (eigenvalues) from principle component analysis. For further information, see legend of Fig. 18.

For the genes contributing to PC1, the factor site showed a significant correlation with gene expression, which differed the most between the two inland sites UX4 and LL3 (Table 6, Fig. 19). Also the genes prominent in PC2 and PC3 showed significant differences in expression associated with the factor site. For PC2, the inland site LL3 differed the most from the other sites in gene expression, whereas for PC3 the inland site

UX4 differed the most. The PCA also revealed a significant difference in temperature response for PC2 related genes (Table 6). Focusing on PC1, the analysis further revealed a categorical difference between sea-exposed and inland sites. However, for PC2 and PC3 there was no categorical difference between habitats. For sea distance and elevation, the PCA revealed that gene expression differed along both gradients in PC1 (Table 6) with a more significant effect of elevation than increasing sea distance on gene expression. This was confirmed by the ANOVA results in the first part, since the cyanobacterial DNA repair genes *radA*, *recF*, *recN* and *recO* (contributing to PC1) showed stronger correlations with the factor elevation than with sea distance. Furthermore, the PCA revealed a significant correlation of temperature response differences with changing elevation for the set of genes contributing to PC1, an effect, which was not evident in the ANOVA approach.

Table 6: Significance (p-values) of multivariate ANOVA for PC1-3. Column 2-4: ANOVA results of linear mixed-effects model using temperature and site as fixed and individual as random factor. Column 5-6: ANOVA results of linear mixed-effects model using temperature and habitat as fixed and site and individual within site as random factors. Column 7-8: temperature and sea-distance as fixed and site and individual as random factors. Column 9-10: temperature and elevation as fixed and site and individual as random factors. Significant effects and/or interactions are marked in grey shading.

1 **PC**	2 **temp.**	3 **site**	4 **interaction temp x site**	5 **habitat**	6 **interaction temp x habitat**	7 **sea distance**	8 **interaction temp x distance**	9 **elevation**	10 **interaction temp x elevation**
1	<0,0001	<0,0001	0,5721	0,0141	0,0926	0,0139	0,0723	0,0007	0,0168
2	<0,0001	<0,0001	0,0162	0,4642	0,3666	0,1205	0,1106	0,4884	0,1078
3	0,0509	0,0006	0,9977	0,1687	0,6967	0,4848	0,6563	0,0188	0,7285

Table 7: Significance (p-values) of Tukey´s honest significant difference test for the effect of the three temperature treatments (5°C vs. 15°C, 5°C vs. 25°C and 15°C vs. 25°C) in PC1-3 (linear mixed-effects model: temperature and site = fixed factors, individual = random factor). Significant values are marked in grey shading.

PC	**5°C vs. 15°C**	**5°C vs. 25°C**	**15°C vs. 25°C**
1	<0,0001	<0,0001	0,4187
2	0,2522	<0,0001	<0,0001
3	0,0463	0,2100	0,7570

3.4 Summary

In conclusion, the analysis using a multivariate ANOVA approach revealed significant expression differences of both fungal and cyanobacterial genes at increased temperatures and between sampling sites where the temperature effect exceeded that of site differences. The differences between sites showed better correlation to the gradually changing variables sea distance and elevation above sea level than to categorical habitats (sea-exposed vs. inland). A subset of fungal and cyanobacterial genes further showed significant differences in temperature response at different sites. However, there were no significant differences in temperature response in correlation with different habitats, sea distances or elevation levels. The correlation analysis and the alternative PCA approach further revealed that certain fungal and cyanobacterial genes were more similar in their expression responses patterns to the investigated factors than others. Whereas the genes contributing mostly to PC1 (fungal *lec2*, cyanobacterial *radA*, *recO*, *recF* and *recN*) showed a major response to the temperature increase from 5°C to 15°C, the majority of other fungal candidates (PC2) were most strongly affected by the increase from 15°C to 25°C, and there was no strongly significant temperature effect associated with PC3 (mainly *lec1*). Generally, the PCA axes of PC1 and PC2 were most strongly influenced by temperature. The PCA also revealed site differences (PC1: greatest in LL3 and UX4; PC2: greatest in LL3; PC3: greatest in UX4) and a difference in temperature response at different sites for the genes contributing to PC2. For PC1, significant habitat differences were found. However, sea distance and elevation again played a more significant role for PC1 and PC3 than for PC2. Generally, gene expression in PC1 was strongly affected by elevation and in PC3 by sea distance. For PC1, the PCA further revealed a significant difference in temperature response with increasing elevation indicating a differing temperature response with increasing elevation.

4 Discussion

This study has shown that expression of certain candidate genes in the fungal and cyanobacterial symbiont of *Peltigera membranacea* lichens changed significantly even at moderately increased temperatures. Therefore, the first research question asking if increased temperatures cause a stress response reflected on the expression level of fungal and *Nostoc* candidate genes was confirmed. Furthermore, the study identified two gene groups, one showing a decrease in expression associated with a temperature shift from 5°C to 15°C and another one characterized by increased expression at 25°C. Expression of cyanobacterial DNA repair genes and the fungal *lec2* gene was downregulated already at 15°C compared to 5°C whereas most fungal heat shock genes, DNA repair genes and genes involved in transcriptional regulation of stress responses were upregulated at 25°C. Gene expression also differed between sites. However the site effect was smaller than the temperature effect. For *lec2* and the cyanobacterial DNA repair genes, the gene expression temperature response also differed significantly between sites. The differences in gene expression showed a greater correlation with increasing sea distance and elevation above sea level than with habitat categories (sea exposed vs. inland). This might be an indication that increasing temperatures might affect lichens from higher elevations in a different way than lichens from lower elevations. This finding contributed to answering the second research question asking if lichens from different habitats differ in temperature response.

4.1 Temperature responses

This study revealed that expression of candidate genes involved in transcriptional regulation of fungal stress responses as well as of several fungal and cyanobacterial genes encoding heat-shock and DNA repair proteins was strongly affected by increasing temperature.

4.1.1 *P. membranacea*

Focusing on the mycobiont *P. membranacea*, three genes involved in the transcriptional regulation of stress-responsive genes (e.g. heat shock and DNA repair genes) showed increased expression at higher temperatures. The TATA-binding protein associated factor *mot1* was significantly upregulated at 25°C. Mot1 is a member of the Swi/Snf2 family and belongs to the SAGA multi-protein complex involved in transcriptional regulation of TATA-box containing genes through repressing the binding of the TATA-binding protein (TBP) (Dasgupta et al. 2007, Lopez-Maury et al. 2008). Under stress conditions, Mot1 is recruited together with the active and assembled SAGA complex and is assumed to control the temporary induction of stress-induced genes, which often

contain the TATA-box element (Zanton and Pugh 2004, Lopez-Maury et al. 2008). Although Mot1 is a repressor of TBP, it has the ability to upregulate gene expression by displacing transcriptionally inactive forms of TBP from TBP-promoters (Sikorski and Buratowski 2009). Therefore, the observed expression patterns of *mot1* might indicate the transcriptional induction of stress-responsive TATA-box containing genes involved in e.g. DNA and protein repair, suggesting that 25°C represent heat stress for *P. membranacea* requiring stress response activation. However, to get a clear and more specific picture the expression analysis of other members of this pathway such as *snf2* and *tbp* would be highly valuable.

Exposure to increased temperatures also caused an upregulation of the fungal histidine kinase *hhk2* gene and the zinc-finger transcription factor *msn2* gene. As part of two-component regulatory systems, histidine kinases such as *hhk2* can function as sensors of environmental stimuli that can trigger a molecular response to environmental stress (Stock et al. 2000, Gasch 2007, Schmoll 2008). In *S. cerevisiae*, a two-component system regulates the Hog1 pathway for transcriptional induction of stress responsive genes under external stress (Schüller et al. 1994, Enjalbert et al. 2006, Gasch 2007). The Hog1-induced transcription factor Msn2 is responsible for activating expression of various stress response genes (heat shock genes, DNA repair genes) containing stress response elements (STREs) (Martínez-Pastor et al. 1996, Rep et al. 2000, Gasch 2007, Liu et al. 2013). Therefore, the observed upregulation of *hhk2* and *msn2* indicates heat stress for the lichens at 25°C leading to the transcriptional upregulation of stress-responsive genes. Secondly, this study confirmed the existence of the conserved Hog1 stress response pathway in *P. membranacea* similar to *S. cerevisiae* and most other fungi, in contrast to the orthologous Sty1-mediated stress response pathway as in *Sz. pombe*, (Gasch 2007). However, for confirmation it should be investigated, whether the expression of *hog1* and other genes contributing to the pathway (see introduction) is also upregulated and whether *hog1* repressing elements are simultaneously downregulated.

In addition to showing increased expression of transcriptional regulators inducing transcription of stress response genes, the study also revealed increased expression of specific candidate genes involved in heat shock and DNA repair. Expression of heat-shock chaperone genes *hsp88* and *hsp98* as well as of the fungal signal peptide peptidase *spp* gene was significantly upregulated at 25°C. Hsp88 and Hsp98 have been described in *Neurospora crassa* (Ascomycota) as important and highly conserved elements of the heat-shock machinery showing marked increase in expression during heat-shock (Vassilev et al. 1992, Plesofsky-Vig and Brambl 1998). Whereas Hsp88 (Hsp110 family) in conjunction with Hsp30 prevents aggregation of denatured proteins during thermal stress, the hexameric Hsp98 (71% identity to *S. cerevisiae* Hsp104) is required for resolubilization/dissociation and refolding of damaged protein aggregates (Vassilev et al. 1992, Plesofsky-Vig and Brambl 1998, Wang et al. 2007, Doyle and Wickner 2009). Following dissociation by Hsp98, the denatured proteins can be refolded in the Hsp40/Hsp70 chaperone system (Doyle and Wickner 2009). Signal peptide peptidases

represent an important quality control for membrane proteins through their ability to remove damaged or misfolded membrane proteins in response to external stress such as heat (Dalbey et al. 2012). In line with these previous results this study indicates that the moderate temperature stress of 15°C to 25°C can represent heat-stress conditions for the lichen-forming fungus *P. membranacea* requiring increased protein repair including dissociation of damaged and aggregated membrane proteins.

The mating-type switching/DNA repair protein Swi10, which plays an important role in DNA repair under stress conditions, and the fungal DNA repair protein UCRNP2_806 also showed increased expression associated with temperature increase. The Swi/Snf2 family member Swi10 has been shown to be involved in nucleotide excision repair under UV-stress conditions (Yasuhira et al. 1999). The gpd-family base-excision DNA repair protein UCRNP2_806 is believed to contribute to base-excision DNA repair (http://www.uniprot.org/uniprot/R1GVL6; accessed 10.07.2015). Therefore, the upregulation of *swi10* and *UCRNP2_806* expression at higher temperatures indicate an increased level of DNA damage calling for repair. However, further studies need to be performed in order to develop a clearer picture of the role of UCRNP2_806, since little is known about its specific pathway or interaction partners.

In addition to genes involved in the fungal stress response, expression of the fungal lectin genes *lec1* and *lec2,* which are thought to be involved in interactions with the *Nostoc* symbiont, was studied to gain information about potential interactions among symbionts at increased temperatures. Most interestingly, gene expression of the fungal lectin *lec2* was strongly downregulated already at a temperature increase to 15°C whereas no significant temperature effect was found for *lec1*. A study on the effect of temperature stress on coral larvae of *Acropora millepora* before they establish the symbiosis with their algal partners showed that mannose-binding C-type lectin in the coral was downregulated at increased temperatures (Rodriguez-Lanetty et al. 2009). This goes hand in hand with our findings concerning *lec2* expression. The carbohydrate binding properties of lectins are important for cell-cell recognition and are therefore though to play an important role in symbiont communication of plant-microbial and fungal-microbial symbioses (McCowen et al. 1986, Sharon and Lis 2004, Antonyuk and Evseeva 2006, Manoharan et al. 2012, Miao et al. 2012). Therefore, the observed drop in *lec2* expression could indicate that increased temperatures might affect the symbiosis between *P. membranacea* and *Nostoc*. In a wheat symbiosis with *Azospirillum brasilense,* different wheat lectin concentrations were shown to affect a range of metabolic processes such as nitrogen fixation in the rhizobacterial symbionts (Antonyuk and Evseeva 2006). Interestingly, the correlation analysis revealed that a set of cyanobacterial DNA repair genes expected to be upregulated in response to heat stress as commonly observed in other bacterial species, showed strong correlation in expression with the fungal *lec2* gene (downregulation in expression) (Fig. 15). This could indicate a relation between the cyanobacterial DNA damage response and the amount of fungal Lec2. The study on wheat lectin revealed that wheat plants that differed in lectin content also had differential stress responses towards nitrogen starvation, indicating a

further role of lectins in the stress response. However, to clarify this relationship and to determine whether a temperature increase could affect the lichen symbiosis itself through changing *lec2* expression, further studies need to be performed. Regarding the fungal lectin gene *lec1*, no temperature-related gene expression differences were found in this study. Miao et al. (2012) showed that the symbiosis-related gene *lec1* is differentially expressed in different lichen tissues (thalli, rhizines and apothecia), which differed by the presence or absence of the photobiont. Depending on the variation intensity it might be conceivable that this tissue-specific variation in expression might have obscured expression changes due to increased temperature even though all samples derived from marginal areas. However, further tissue-specific gene expression studies need to be performed in order to fully understand differential expression of *lec1*.

4.1.2 *Nostoc*

In the photobiont *Nostoc*, expression of the heat-shock chaperone genes *hsp90* and *dnaJ* as well as the cyanobacterial gene *Npun_F4482* was significantly upregulated at higher temperatures. Hsp90 prevents non-native proteins - mainly signal transduction proteins - from forming unproductive protein aggregates (Wiech et al. 1992, Hartl 1996) and contributes to general refolding of denatured proteins under heat stress (Hartl 1996, Young 2001). Similarly, as part of the Hsp70/Hsp40 cycle, the Hsp40 protein DnaJ contributes to refolding of denatured proteins due to heat and cold stress (Langer et al. 1992, King-Chuen and Wai 1998). Studies among fish, plants and cyanobacteria revealed increased gene expression of both *hsp90* and *dnaJ* under heat and oxidative stress (Hossain and Nakamoto 2003, Reddy et al. 2011). Confirming these findings, the present study suggests that 25°C also induces heat stress in the *Nostoc* symbiont. Npun_F4482 is a helicase-domain-containing protein identified in *Nostoc punctiforme,* showing similarity to DNA-repair protein Rad25 in eukaryotes. Eukaryotic Rad25 has been shown to function in nucleotide-excision repair processes of damaged DNA under UV stress. Therefore, the observed increase in expression could indicate elevated DNA damage at higher temperatures calling for repair in *Nostoc*. However, further studies need to be performed in order to determine in which DNA repair pathway Npun_F4482 might be involved and why it behaves in a different manner than the other *Nostoc* DNA repair genes studied (see below).

In contrast to what was expected, expression of heat-shock gene *groEL* and the LexA-regulated SOS DNA repair genes *radA, recO, recN* and *recF* was not upregulated at increased temperatures. Together with GroES, GroEL is part of the Hsp60/Hsp10 complex for protein damage repair (Goloubinoff et al. 1997, Melkani et al. 2005). In *E. coli* and *B. subtilis,* the RecN protein is recruited for repair of DNA double strand breaks followed by the recruitment of RecF and RecO and further RadA (Sargentini and Smith 1986, Odsbu and Skarstad 2014). The *groEL* downregulation contrasts to studies showing an upregulation of *groEL* in response to heat shock in *E. coli* and *Anabaena* (Mendoza et al. 1996, Melkani et al. 2005, Rajaram and Apte 2010). Expression of *groEL* and *groES* is

regulated by the transcriptional repressor HrcA. When *hrcA* is downregulated, *groEL* and *groES* expression can be induced (Rajaram and Apte 2010, Wallenius et al. 2011). This negative expression correlation can be confirmed in this study, since *hrcA* expression shows a strongly significant increase already at 15°C. This result indicates that in contrast to the heat stress system involving Hsp90 and DnaJ, the GroEL/GroES heat stress response system is repressed in the *Nostoc* photobiont at increased temperatures. However, to further strengthen this finding, it would be necessary to also analyze expression of the *groES* and *hrcA* genes using lichen thalli and cultured *Nostoc* strains from lichens. Furthermore, genome analysis of symbiotic *Nostoc* in Icelandic lichens has revealed two copies of the *groEL* gene. Therefore, the temperature response of the second *groEL* copy also needs to be determined. The downregulation of the SOS DNA repair genes is in contrast to previous studies on these genes in other bacteria (Sargentini and Smith 1986, Rostas et al. 1987, Odsbu and Skarstad 2014) and to the expression results for the fungal DNA repair genes investigated (e.g. *UCRNP2_806*, *swi10*), which were found to be upregulated at increased temperatures. One possible explanation could be that the observed downregulation is due to a general downregulation of metabolism and physiology in *Nostoc* (Kosugi et al. 2014) in order to prepare for the usual drying out that occurs at increasing temperatures. However, this hypothesis is unlikely, since in that case expression downregulation of more genes involved in all other kinds of processes would have been expected, including the cyanobacterial reference genes *rnpB* and *secA* (which remained stable in expression). Another explanation could be that the observed expression results might be related to the temperature exposure time. Studies on UV stress in *E. coli* revealed that there are two phases after stress induction. In the first phase, which lasts about 10-20 minutes, the nucleoids are aggregated into compact structures in the cell and in the second phase, which lasts about one hour, the DNA is distributed in the whole cell. Expression of *recN* and *recO* and also expression of the recombination proteins *recA* and *recR* was found to be upregulated in the first phase after induction of the SOS response to DNA damage (Odsbu and Skarstad 2014). Since in this study expression was measured after three hours of exposure to the increased temperature, the expression might already have dropped after primary induction. This could explain the observed decrease in expression of DNA repair genes in *Nostoc*. In order to determine whether DNA repair gene expression in *Nostoc* increases transiently after stress induction, a time series needs to be performed where RNA sampling is performed at several timepoints within the first and the second phase of the DNA damage response. An alternative hypothesis explaining the expression downregulation of the DNA repair genes and perhaps also of the groEL gene involves a possible interaction between the symbionts. As mentioned before, the multiple regression analysis revealed a strong positive correlation in expression of the cyanobacterial DNA repair genes with fungal *lec2* expression. This could be an indication that the observed downregulation of the DNA repair genes is related to the drop in *lec2* expression. However, to further investigate this hypothesis, the relation between *lec2* and the cyanobacterial SOS DNA repair system (including also other candidates involved in the SOS DNA repair such as *recA/B/C/J, radB* and repressor *lexA*) as well as possibly the GroEL/GroES heat shock system needs to be determined further. In addition, DNA repair

gene and *groEL/groES* expression should be determined in pure *Nostoc* cultures for comparison, since a difference between pure *Nostoc* and *Nostoc* living in symbiosis could support a possible symbiosis effect.

4.1.3 Summary of temperature responses

In summary, this study has revealed that a temperature increase from 5°C to 15°C and further to 25°C leads to transcriptional induction of diverse stress response pathways reflected in the upregulation of gene expression of many stress response genes in *P. membranacea*. A subset of the fungal candidate genes are known to play an important role in processes such as the heat stress response e.g. through upregulating the transcription of heat stress-responsive genes (*mot1, hhk2, msn2*) (Dasgupta et al. 2007, Gasch 2007, Lopez-Maury et al. 2008, Schmoll 2008, Liu et al. 2013) or through direct repair of heat stress-induced protein damage or misfolding (*hsp88, hsp98*) (Vassilev et al. 1992, Plesofsky-Vig and Brambl 1998) or DNA damage (*UCRNP2_806*, *swi10*). Expression of most of the fungal stress response genes was increased at 25°C compared to 5°C and 15°C. Hence, 25°C represents a heat-stress condition for Icelandic *P. membranacea*. However, to further strengthen the results, expression of other genes involved in the described heat-stress responses and DNA repair pathways need to be investigated. Candidate genes of interest would be: the fungal heat shock genes *hsp30*, *hsp40* and *hsp70*, further members of the Swi/Snf2 pathway (e.g. *snf2* and *tbp*) as well as further members of the Hog1 pathway (Hog1, Sln1, Ssk1, Ssk2, Ssk22, phosphatases Pyp1 and Pyp2 and the transcriptional regulator Msn4).

For the photobiont *Nostoc*, the results also showed an upregulation of candidate genes involved in cyanobacterial heat stress response processes (*dnaJ, hsp90*) and DNA repair (*Npun_F4482*). Hence, the tested temperatures (15°C and 25°C) also seem to cause heat stress in the photosymbiont. However, expression of cyanobacterial SOS DNA repair genes (*radA, recF, recN* and *recO*) and heat shock gene *groEL* was significantly downregulated at increased temperatures, which contrasts to other studies in bacteria. The drop in *groEL* expression makes sense in a biological context, since expression of the *groEL* repressor gene *hrcA* is upregulated at increased temperatures. However, expression of a second copy of the *groEL* gene present in *Nostoc* needs to be investigated to strengthen these findings. For the expression drop of the *Nostoc* SOS DNA repair genes an explanation might be that the gene expression quantification after three hours of exposure only captured the second stress response phase characterized by decreased expression after an initial increase. Sampling at several timepoints during the heat exposure could help to resolve this question. Taking the positive correlation of the drop of cyanobacterial DNA repair gene expression with the downregulation of fungal lectin *lec2* into account, an alternative explanation could be a possible functional relation. However, the connection between the fungal *lec2* and the bacterial genes needs to be investigated further (e.g. by a comparison with the expression in pure *Nostoc* cultures) in order to clarify the role of *lec2* in establishing and maintaining or changing the symbiosis and to elucidate possible other

roles. Among additional *Nostoc* candidate genes involved in the described pathways are the cyanobacterial heat shock gene *groES*, the cyanobacterial DNA repair genes *recA/B/C/J, radB* and the repressor of the SOS DNA damage response, *lexA*.

A study on *P. scabrosa* (MacFarlane and Kershaw 1980) determined that 25°C represents a thermal stress condition for the lichen reflected in nitrogenase activity, photosynthesis and respiration rate. Generally, the photobiont showed a lower stress tolerance than the photobiont. The results of the present study go hand in hand with this previous findings since many photobiont genes showed a stress response already at lower temperatures (15°C) than most mycobiont genes (25°C).

4.2 Habitat differences

4.2.1 Sites

To enable short-term acclimation to a common environment, all samples were kept in the laboratory for three weeks at constant temperature, light and moisture conditions. This should minimize differences due to long-term acclimatization to naturally differing environmental conditions. This acclimation method seemed to have been successful since a short-term response in gene expression after increased temperatures was clearly detectable. However, in addition to the expression changes detected with increasing temperature, gene expression also differed significantly between sampling sites, especially the two inland sites located at Uxahryggir (UX4) and Hrauneyjar (LL3) as clearly revealed by the PCA. Since all samples had been acclimated to constant conditions, these observed differences could either be a long-term site acclimatization effect and/or local adaptation (genetic) of the sampled lichen populations to the different environments. Genetic variation between populations is a well-known phenomenon studied in many species (Iguchi et al. 2004, Pálsson et al. 2014) but has not yet found major attention in connection with lichens. One study on the lichen *Tephromela atra* in Europe found genetic differences between individuals from wood vs. rock substrates within sites (Muggia et al. 2008). Another study on *Ramalina menziesii* showed genetic variation in the photobiont according to phorophyte species (Werth and Sork 2010). However, to further determine the role of long-term acclimatization vs. genetic adaptation effects in this study, the genetic background of the lichen individuals has to be taken into account. RAD sequencing has been performed as part of another project, which will provide genotype data of samples included in this study that can be used to address this question. For an evaluation of expression differences due to site-specific acclimation or genetic differences, it would be interesting to repeat the experiment using lichen individuals from several sites, transplanting them to the same habitat for a whole-year acclimation cycle (common garden) and comparing gene expression results of this experiment with the present results.

4.2.2 Categories and gradients

In addition to differences between individual study sites, the PCA revealed a general difference in gene expression between categorical seashore versus inland habitats for the fungal *lec2* gene and a subset of cyanobacterial DNA repair genes (*radA*, *recF*, *recN* and *recO*) (PC1). This indicates not only acclimation to or genetic differences between local habitats but also a categorical higher-level acclimatization dependent on shore proximity or inland location, although this effect was much weaker than the temperature response. However, in the above gene set, local expression changes were much more strongly correlated with increasing elevation above sea level than with categorical habitats (sea-exposed vs. inland). In addition, expression patterns of the genes mentioned above (PC1) and for the fungal *lec1* gene also correlated significantly with gradually increasing distance from the sea. Hence, the relation between variation in gene expression and habitat cannot be explained just by the categorical sea proximity or inland location but might be more complex, depending on gradually changing variables such as sea distance, elevation or potentially other confounding climatic parameters. A relationship between genetic variation and a gradually changing physical parameter such as altitude has been found for many species. A 2008 study showed differential expression of nearly 200 transcripts between high-altitude and low-altitude populations of the rufous-collared sparrow (*Zonotrichia capensis*) (Cheviron et al. 2008). However, in our system, other abiotic and biotic factors such as soil properties, topology, nutrient availability, salt content, microclimatic effects such as irradiation (e.g. presence/absence of tree cover) or niche competition can also explain gene expression differences between local populations.

4.2.3 Temperature response

Focusing on the temperature response in gene expression, response differences between sites were found for the majority of fungal candidate genes (PC2). For the PC1 genes (*Nostoc* DNA repair genes and fungal *lec2*), there were response differences associated with increasing elevation above sea level. This could be an indication that long-term acclimatization or genetic adaptation of a lichen population to a specific location and along an elevation gradient affects the reaction of its individuals to increasing temperature conditions at the gene expression level. Differences in response to salt stress were found in coastal vs. inland populations of the monkey flower (*Mimulus guttatus*) and the Mediterranean shrub *Atriplex halimus* (Ben Hassine et al. 2008, Lowry et al. 2009). In the brown trout (*Salmo trutta L.*) expression differences of common heat-shock and osmoregulative genes were found between individuals from different populations correlated to local adaptation on a regional scale (Larsen et al. 2008). In 1989, a study on the epiphytic lichen *Ramalina menziesii* revealed distinct differences in photosynthetic and respirational responses at increasing temperatures between lichens from locations with different natural temperature variation (Larson 1989). In this study, individuals from locations with naturally higher temperature stress (greater temperature amplitude) also showed a higher stress resistance in the temperature treatments compared to individuals

from cool coastal rainforest areas. Similar to these studies, the findings of the current study on Icelandic *P. membranacea* lichens suggests that lichen populations from different locations along an elevation gradient differ slightly in their stress responses to moderately increased temperatures due to long-term acclimatization, or due to genetic differences.

5 Conclusion

The aims of this study were to determine stress responses in Icelandic *P. membranacea* lichens to moderately increased temperatures and possible response differences due to differing habitats. Therefore, lichens from sea-exposed and inland locations were collected and expression of fungal and cyanobacterial candidate genes (involved in heat stress response and DNA repair) was investigated at 5°C, 15°C and 25°C. The results indicated that increased temperatures of 15°C and 25°C represent a heat stress condition for Icelandic populations of the lichen forming fungus *P. membranacea* and for their *Nostoc* photobionts. Higher temperatures led to increased expression of genes involved in transcriptional induction of diverse fungal stress response pathways and of genes directly involved in heat shock-induced protein and DNA damage repair in both symbionts. However, a set of photobiont SOS DNA repair genes (*radA*, *recF*, *recN* and *recO*), which was strongly correlated in expression with the fungal galectin-like gene *lec2*, was downregulated when brought from 5°C to 15°C. Since *lec2* is thought to play an important role in the symbiosis, this finding is an interesting indication for a possible interplay between symbionts and should be investigated further. Generally, the PCA analysis added some information to the results, which would not have been pointed out clearly by the ANOVA approach and has therefore proven very useful in this context. The PCA revealed two gene groups with differing temperature response, the PC2 fungal genes affected at 25°C and the PC1 cyanobacterial DNA repair genes and *lec2* showing decreased expression at 15°C. However, to support a possible symbiosis effect, it needs to be clarified further whether the photobiont cultured in isolation shows similar gene expression patterns at increased temperatures as in the symbiotic state. Furthermore, the study suggests a set of additional candidate genes involved in general stress response pathways of fungi and bacteria, which can be investigated in the future to strengthen the findings on heat stress presented here.

The conduction of a three-week acclimation phase has proven useful in minimizing individual differences due to site-specific acclimatization to temperature, light, moisture or other unknown factors potentially differing between habitats. Expression responses to increased temperatures in the laboratory were clearly detectable. However, the study also revealed site-specific expression differences that could be either due to local long-term acclimatization or genetic adaptation. For a larger number of fungal and cyanobacterial genes, these differences had a stronger correlation with gradually increasing sea distance and elevation than with general habitats categorized by either sea proximity or inland location showing the complexity of stress response variation. For the PC1 gene group (see above), the study further revealed differences in the temperature response associated with gradually increasing elevation. Hence, this study suggests that Icelandic lichen populations

might differ in their response to increased temperatures depending on the elevation of their habitat and might therefore be affected differently by globally increasing temperatures. However, further studies need to be performed including more individuals and sites along an elevation gradient to allow a more precise evaluation of the elevation effect. In addition to an elevation gradient there are also other factors (e.g. tree coverage, salt content) that might affect thermal stress responses in a species, which need to be investigated further in order to receive a more general picture of the flexibility of temperature stress responses in lichens.

As a next step, it would be interesting to also investigate long-term temperature responses in lichens since this study was mainly focused on short-term acclimation effects in gene expression in response to increased temperatures. An elevation of stress responsive candidate gene expression at various timepoints beyond three hours (e.g. up to three weeks) could provide information in a longer time frame. In other species different phases of temperature stress response have been observed, characterized by up- or downregulations of certain gene groups (O Leyva-Perez et al. 2015). Such information would be important for a more complete answer to the general question, if and how lichens are able to survive constant temperature stress and if there are consistent differences between the symbiosis partners in coping with long-term temperature stress. The further investigation of the relation of the fungal lectin *lec2* and cyanobacterial genes that showed correlated expression could provide further information about how increased temperatures may affect the symbiotic interaction of *P. membranacea* and *Nostoc*. However, for a general picture of long-term temperature responses in lichens it is essential to differentiate between acclimatization and adaptation effects by making use of the genotyping data and by reverting acclimatizations through transplantation of thalli. Only if achieved stress adaptability can be inherited to the next generation, lichens may be able to avoid extinction in times of increasing stress levels due to changing climate.

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Appendix A

Protocols

RNeasy® Plant Mini Kit (cat. no. 74904)

Notes before starting:

- Add 10 µl 1-mercaptoethanol (.-ME) to 1 ml Buffer RLT before use. Buffers .ME can be stored at room temperature for up to 1 month.
- Add 4 volumes of ethanol (96–100%) to Buffer RPE for a working solution.

Procedure:

1. Take a piece of marginal tissue (5 x 5 mm) from each sample stored in RNA later after each temperature treatment After three-hour temperature treatment into a new 2.0 ml tube with cap (Sarstedt, Ref. 72.694) and add 450 .l Buffer RLT and one metal beat.
2. Homogenize sample for 4 minutes in Mini-BeadBeater-16 (BioSpec Products, Cat. Nr. 706).
3. Transfer the lysate to a QIAshredder spin column (lilac) placed in a 2 ml collection tube. Centrifuge for 2 min at full speed. Transfer the supernatant of the flow-through to a new microcentrifuge tube (not supplied) without disturbing the cell-debris pellet.
4. Add 0.5 volume of ethanol (96–100%) to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 5.
5. Transfer the sample (usually 650 .l), with any precipitate, to an RNeasy Mini spin column (pink) in a 2 ml collection tube (supplied). Close the lid, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flowthrough.
6. Add 700 .l Buffer RW1 to the RNeasy spin column. Close the lid, and centrifuge for 15 s at ≥8000 x g. Discard the flow-through.
7. Add 500 .l Buffer RPE to the RNeasy spin column. Close the lid, and centrifuge for 15 s at ≥8000 x g. Discard the flow-through.

8. Add 500 .l Buffer RPE to the RNeasy spin column. Close the lid, and centrifuge for 2 min at ≥8000 x g. Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied). Centrifuge at full speed for 1 min to dry the membrane.

9. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 .l RNase-free water directly to the spin column membrane. Close the lid, and centrifuge for 1 min at ≥8000 x g to elute the RNA.

10. If the expected RNA yield is >30 .g, repeat step 9 using another 30–50 .l of RNase-free water. Alternatively, use the eluate from step 9 (if high RNA concentration is required). Reuse the collection tube from step 9. For up-to-date licensing information and productspecific disclaimers, see the respective QIAGEN kit handbook or user manual.

11. Store at -80°C

DNase 1 digestion (New England Biolabs)

1. Measure RNA concentration in each sample using NanoDrop® Spectrophotometer (Thermo Scientific, Waltham, USA)

2. Prepare the following reaction for each sample in a 1,5 ml microcentrifuge tube:

    - 5 µl reaction buffer
    - 4 µl DNase 1 (1000 U/µl)
    - x µl RNA (for a final concentration of 50 ng/µl)
    - x µl RNase-free water (to fill up to 50 µl)
      Total reaction volume = 50 µl

3. Mix gently

4. Incubate at 37°C for 15 min

5. Heat-inactivate DNase I by incubation at 75°C for 5-10 min

6. Cool on ice & continue with quality control (NanoDrop) to adjust final RNA concentration

7. Store at -80°C

cDNA synthesis (High Capacity cDNA Reverse Transcription Kit (Appl. Biosyst.))

1. Prepare mastermix for each reaction:

   2 µl reaction buffer
   2 µl Random Primers/Hexamers
   0,8 µl dNTP mix (2.5 mM)
   4,2 µl RNase free water

2. Add 9 µl of mastermix in each reaction tube and 10 µl of RNA sample (= 500 ng RNA)
3. Add 1 µl Reverse Transcriptase (1 µl RNase free water for -RT controls)
   → Total reaction volume 20 µl
4. Perform PCR reaction with the following steps:

   Incubate mixture at 25°C for 10 min
   Incubate mixture at 37°C for 120 min
   Incubate mixture at 85°C for 5 min to stop the reaction

5. Dilute samples with RNase-free H_2O to a final concentration of 10 ng/µl
6. Store product at -20°C

Quantitative real-time PCR (RT-qPCR, SYBR Green method, 7500 qPCR system (Appl. Biosyst.)

Reaction mix

SYBR Green® Mix	5,0 µl
forward primer (5 µM)	0,5 µl
reverse primer (5 µM)	0,5 µl
RNase-free water	3,0 µl
diluted cDNA template (10ng/µl)	1,0 µl

Temperature cycle

Holding stage:	50°C	2 min.
	95°C	10 min.
Cycling Stage:	95°C	15 sec.
	60°C	1 min.

40 cycles

Temperature data

Table S1: Daily mean temperatures and standard deviations at the sampling sites RY7 and HF2 (sea-exposed), as well as at LA1, HV3, and LL3 (inland) in June 2013. Mean temperature was calculated for every day based on hourly measurements.

Date	RY7 [°C]	HF2 [°C]	LA1 [°C]	HV3 [°C]	LL3 [°C]
	Day Mean ± SD	Day Mean ± SD	Day Mean ± SD	Day Mean ± SD	Day Mean ± SD
01.06.13	13,55 ± 4,21	10,01 ± 2,62	9,87 ± 3,95	12,01 ± 4,60	7,83 ± 2,60
02.06.13	16,44 ± 8,26	13,26 ± 3,91	12,60 ± 3,44	16,92 ± 7,19	13,06 ± 5,12
03.06.13	10,24 ± 0,90	11,73 ± 1,49	10,59 ± 1,03	15,33 ± 5,38	13,69 ± 3,44
04.06.13	10,11 ± 1,15	10,20 ± 0,71	10,31 ± 1,43	14,45 ± 5,29	15,76 ± 6,93
05.06.13	11,72 ± 2,72	13,36 ± 1,84	18,49 ± 6,25	22,93 ± 8,00	20,61 ± 7,41
06.06.13	12,01 ± 2,00	13,89 ± 2,96	13,70 ± 3,14	18,27 ± 5,31	16,95 ± 6,57
07.06.13	13,65 ± 4,08	13,51 ± 3,04	12,29 ± 2,52	18,96 ± 7,64	14,82 ± 4,46
08.06.13	14,31 ± 3,21	13,01 ± 3,25	12,82 ± 2,08	16,83 ± 5,56	16,45 ± 6,45
09.06.13	11,41 ± 1,68	13,61 ± 3,41	14,76 ± 3,89	15,08 ± 4,60	12,03 ± 3,28
10.06.13	11,05 ± 1,12	14,61 ± 3,13	15,67 ± 4,06	18,55 ± 6,20	13,16 ± 2,33
11.06.13	10,31 ± 1,13	14,42 ± 3,03	10,47 ± 1,32	12,55 ± 2,87	10,46 ± 1,66
12.06.13	15,09 ± 3,77	14,39 ± 3,02	15,04 ± 4,20	20,71 ± 8,37	18,13 ± 9,53
13.06.13	15,05 ± 2,20	15,20 ± 3,12	13,32 ± 3,30	14,02 ± 4,19	12,94 ± 4,99
14.06.13	16,52 ± 3,27	14,67 ± 2,62	16,86 ± 5,89	21,93 ± 9,54	22,88 ± 11,36
15.06.13	15,12 ± 3,52	13,77 ± 3,04	16,07 ± 6,73	25,77 ± 10,39	22,60 ± 10,32
16.06.13	14,47 ± 3,63	11,17 ± 1,50	10,66 ± 1,48	13,92 ± 3,15	13,88 ± 5,18
17.06.13	15,78 ± 6,60	12,42 ± 2,56	12,41 ± 4,19	17,99 ± 7,66	15,01 ± 5,09
18.06.13	11,40 ± 2,87	11,39 ± 2,14	11,16 ± 2,76	14,27 ± 5,53	11,56 ± 3,49
19.06.13	18,68 ± 6,89	13,36 ± 3,58	15,67 ± 4,67	16,52 ± 7,75	13,59 ± 7,97
20.06.13	24,08 ± 10,94	13,89 ± 2,86	19,64 ± 7,66	24,74 ± 10,95	18,76 ± 8,46
21.06.13	25,26 ± 13,12	16,02 ± 3,60	20,70 ± 9,05	22,08 ± 9,74	15,76 ± 7,04
22.06.13	17,36 ± 5,42	15,71 ± 3,03	18,71 ± 5,39	22,65 ± 9,08	16,60 ± 6,35
23.06.13	17,84 ± 6,45	14,86 ± 3,02	21,77 ± 8,26	26,24 ± 10,93	24,54 ± 12,05
24.06.13	12,95 ± 2,54	16,08 ± 3,17	15,54 ± 5,92	20,96 ± 8,61	21,48 ± 10,49
25.06.13	15,97 ± 6,36	14,58 ± 4,75	15,57 ± 5,61	19,43 ± 8,39	11,75 ± 2,48
26.06.13	13,84 ± 6,77	11,64 ± 2,43	10,59 ± 2,62	14,04 ± 7,00	10,96 ± 4,35
27.06.13	9,65 ± 0,92	9,13 ± 0,66	7,31 ± 0,53	10,10 ± 1,64	8,21 ± 1,99
28.06.13	15,01 ± 3,29	10,80 ± 2,06	13,03 ± 5,01	16,75 ± 5,20	10,49 ± 3,28
29.06.13	18,34 ± 8,72	14,58 ± 1,62	14,90 ± 3,75	17,31 ± 4,12	13,63 ± 3,57
30.06.13	25,12 ± 10,55	15,44 ± 2,91	20,01 ± 7,41	21,18 ± 6,50	18,54 ± 6,83

Statistical data

Raw data

Table S2: RT-qPCR raw data. Ct values for all fungal and cyanobacterial reference and candidate genes for every habitat, site, sea distance [km], elevation [m], temperature and biological replicate

→ attached as additional file

Reference gene variation

Table S3: Reference gene variation. Mean and SD (and variation in %) of all Ct values for fungal and cyanobacterial reference genes (see table S2).

Species	Ref. gene	Ct Mean	SD	Variation [%]
Pmem	**gpd1**	20,09043	1,529958	7,62
	tub2	20,55559	1,889016	9,19
Nostoc	**rnpB**	15,99485	2,231824	13,95
	secA	21,96816	1,901631	8,66

ANOVA results

Table S4: Detailed ANOVA output for linear mixed effects model with “site” and “temperature” as fixed and “individual” as random factor.

Species	Gene	factor	numDF	denDF	F-value	p-value
Pmem	lec2	(Intercept)	1	92	193,881	<0,0001
		temp	2	92	25,936	<0,0001
		site	7	92	6,181	<0,0001
		temp:site	14	92	0,603	0,8560
Pmem	lec1	(Intercept)	1	92	295,016	<0,0001
		temp	2	92	0,530	0,5903
		site	7	92	9,251	<0,0001
		temp:site	14	92	0,537	0,9046
Pmem	swi10	(Intercept)	1	92	6.142,159	<0,0001
		temp	2	92	22,050	<0,0001
		site	7	92	13,036	<0,0001
		temp:site	14	92	1,513	0,1218
Pmem	spp	(Intercept)	1	92	1.523,477	<0,0001
		temp	2	92	14,807	<0,0001
		site	7	92	20,504	<0,0001
		temp:site	14	92	0,858	0,6052
Pmem	hhk2	(Intercept)	1	92	851,220	<0,0001
		temp	2	92	77,218	<0,0001

		site	7	92	4,605	0,0002
		temp:site	14	92	2,296	0,0095
Pmem	mot1	(Intercept)	1	92	905,156	<0,0001
		temp	2	92	19,570	<0,0001
		site	7	92	26,676	<0,0001
		temp:site	14	92	1,999	0,0261
Pmem	msn2	(Intercept)	1	92	42,191	<0,0001
		temp	2	92	95,204	<0,0001
		site	7	92	17,396	<0,0001
		temp:site	14	92	1,742	0,0602
Pmem	UCRNP2_806	(Intercept)	1	92	9.729,700	<0,0001
		temp	2	92	19,484	<0,0001
		site	7	92	9,917	<0,0001
		temp:site	14	92	2,564	0,0037
Pmem	hsp98	(Intercept)	1	92	1.351,241	<0,0001
		temp	2	92	96,564	<0,0001
		site	7	92	16,179	<0,0001
		temp:site	14	92	1,968	0,0290
Pmem	hsp88	(Intercept)	1	92	1.622,327	<0,0001
		temp	2	92	159,863	<0,0001
		site	7	92	22,992	<0,0001
		temp:site	14	92	2,388	0,0069
Nostoc	hrcA	(Intercept)	1	92	2.702,389	<0,0001
		temp	2	92	36,794	<0,0001
		site	7	92	4,974	<0,0001
		temp:site	14	92	1,960	0,0297
Nostoc	Npun_F4482	(Intercept)	1	92	14.382,552	<0,0001
		temp	2	92	19,039	<0,0001
		site	7	92	15,056	<0,0001
		temp:site	14	92	0,725	0,7440
Nostoc	hsp90	(Intercept)	1	92	1.961,028	<0,0001
		temp	2	92	66,983	<0,0001
		site	7	92	5,149	0,0001
		temp:site	14	92	0,588	0,8684
Nostoc	dnaJ	(Intercept)	1	92	3.106,882	<0,0001
		temp	2	92	72,992	<0,0001
		site	7	92	6,237	<0,0001
		temp:site	14	92	1,072	0,3926
Nostoc	groEL	(Intercept)	1	92	115,761	<0,0001
		temp	2	92	7,072	0,0014
		site	7	92	6,096	<0,0001
		temp:site	14	92	0,533	0,9073
Nostoc	recO	(Intercept)	1	92	3.761,638	<0,0001
		temp	2	92	36,989	<0,0001
		site	7	92	1,772	0,1023

		temp:site	14	92	1,885	0,0380
Nostoc	recN	(Intercept)	1	92	5.534,519	<0,0001
		temp	2	92	20,754	<0,0001
		site	7	92	10,710	<0,0001
		temp:site	14	92	1,007	0,4531
Nostoc	recF	(Intercept)	1	92	10.592,050	<0,0001
		temp	2	92	39,002	<0,0001
		site	7	92	7,856	<0,0001
		temp:site	14	92	0,358	0,9828
Nostoc	radA	(Intercept)	1	92	9.940,102	<0,0001
		temp	2	92	180,677	<0,0001
		site	7	92	6,127	<0,0001
		temp:site	14	92	2,007	0,0254

Table S5: Detailed Tukey test ouput for linear mixed effects model with „temperature“ and „site“ as fixed and „individual“ as random factors for single temperature comparision.

Species	**Gene**	**contrast**	**estimate**	**SE**	**df**	**t.ratio**	**p.value**
Pmem	hsp88	5°C vs. 15°C	-0,2516717	0,1377392	92	-1.827	0,1665
		5°C vs. 25°C	1,9959355	0,1377392	92	14.491	<0,0001
		15°C vs. 25°C	2,2476072	0,1377392	92	16.318	<0,0001
Pmem	hsp98	5°C vs. 15°C	-0,4861585	0,1952059	92	-2.490	0,0383
		5°C vs. 25°C	2,0682280	0,1952059	92	10.595	<0,0001
		15°C vs. 25°C	2,5543864	0,1952059	92	13.086	<0,0001
Pmem	UCRNP2_806	5°C vs. 15°C	0,4053310	0,1609818	92	2.518	0,0358
		5°C vs. 25°C	0,9990094	0,1609818	92	6.206	<0,0001
		15°C vs. 25°C	0,5936785	0,1609818	92	3.688	0,0011
Pmem	msn2	5°C vs. 15°C	-0,1358597	0,1542843	92	-0,881	0,6539
		5°C vs. 25°C	1,7720295	0,1542843	92	11.485	<0,0001
		15°C vs. 25°C	1,9078892	0,1542843	92	12.366	<0,0001
Pmem	mot1	5°C vs. 15°C	0,2230725	0,1496452	92	1.491	0,3001
		5°C vs. 25°C	0,8989682	0,1496452	92	6.007	<0,0001
		15°C vs. 25°C	0,6758957	0,1496452	92	4.517	0,0001
Pmem	hhk2	5°C vs. 15°C	-0,7683285	0,1467146	92	-5.237	<0,0001
		5°C vs. 25°C	1,0477709	0,1467146	92	7.142	<0,0001
		15°C vs. 25°C	1,8160993	0,1467146	92	12.378	<0,0001
Pmem	spp	5°C vs. 15°C	0,2264704	0,1801568	92	1.257	0,4230
		5°C vs. 25°C	0,9393115	0,1801568	92	5.214	<0,0001
		15°C vs. 25°C	0,7128411	0,1801568	92	3.957	0,0004
Pmem	swi10	5°C vs. 15°C	0,3485511	0,1321818	92	2.637	0,0263
		5°C vs. 25°C	0,8719622	0,1321818	92	6.597	<0,0001
		15°C vs. 25°C	0,5234111	0,1321818	92	3.960	0,0004
Pmem	lec1	5°C vs. 15°C	-0,1277836	0,4466479	92	-0,286	0,9559
		5°C vs. 25°C	0,3187378	0,4466479	92	0,714	0,7561
		15°C vs. 25°C	0,4465214	0,4466479	92	1.000	0,5789

Pmem	lec2	5°C vs. 15°C	-3,4173021	0,5519744	92	-6.191	<0,0001
		5°C vs. 25°C	-3,4678366	0,5519744	92	-6.283	<0,0001
		15°C vs. 25°C	-0,0505345	0,5519744	92	-0,092	0,9954
Nostoc	radA	5°C vs. 15°C	-1,5053560	0,1397182	92	-10.774	<0,0001
		5°C vs. 25°C	-2,6476620	0,1397182	92	-18.950	<0,0001
		15°C vs. 25°C	-1,1423060	0,1397182	92	-8.176	<0,0001
Nostoc	recF	5°C vs. 15°C	-0,2291281	0,1823015	92	-1.257	0,4232
		5°C vs. 25°C	-1,4947433	0,1823015	92	-8.199	<0,0001
		15°C vs. 25°C	-1,2656153	0,1823015	92	-6.942	<0,0001
Nostoc	recN	5°C vs. 15°C	-0,5504729	0,1162681	92	-4.735	<0,0001
		5°C vs. 25°C	-0,7152133	0,1162681	92	-6.151	<0,0001
		15°C vs. 25°C	-0,1647404	0,1162681	92	-1.417	0,3365
Nostoc	recO	5°C vs. 15°C	-0,8864771	0,1738128	92	-5.100	<0,0001
		5°C vs. 25°C	-1,4857445	0,1738128	92	-8.548	<0,0001
		15°C vs. 25°C	-0,5992674	0,1738128	92	-3.448	0,0024
Nostoc	groEL	5°C vs. 15°C	-0,4133553	0,2866424	92	-1.442	0,3238
		5°C vs. 25°C	-1,0688768	0,2866424	92	-3.729	0,0010
		15°C vs. 25°C	-0,6555215	0,2866424	92	-2.287	0,0627
Nostoc	dnaJ	5°C vs. 15°C	0,2135025	0,1216253	92	1.755	0,1905
		5°C vs. 25°C	1,3658898	0,1216253	92	11.230	<0,0001
		15°C vs. 25°C	1,1523873	0,1216253	92	9.475	<0,0001
Nostoc	hsp90	5°C vs. 15°C	1,8272570	0,2017396	92	9.058	<0,0001
		5°C vs. 25°C	2,1726162	0,2017396	92	10.769	<0,0001
		15°C vs. 25°C	0,3453592	0,2017396	92	1.712	0,2062
Nostoc	Npun_F4482	5°C vs. 15°C	0,5887914	0,1257017	92	4.684	<0,0001
		5°C vs. 25°C	0,7317039	0,1257017	92	5.821	<0,0001
		15°C vs. 25°C	0,1429126	0,1257017	92	1.137	0,4940
Nostoc	hrcA	5°C vs. 15°C	0,4505558	0,128221	92	3.514	0,0020
		5°C vs. 25°C	1,0942596	0,128221	92	8.534	<0,0001
		15°C vs. 25°C	0,6437038	0,128221	92	5.020	<0,0001

Table S6: Detailed ANOVA output for linear mixed effects model with „habitat“ and „temperature“ as fixed and „site“ and „individual“ as random factors.

Species	**Gene**	**factor**	**numDF**	**denDF**	**F-value**	**p-value**
Pmem	lec2	(Intercept)	1	76	226,958	<0,0001
		temp	2	76	44,737	<0,0001
		habitat	1	6	15,745	0,0074
		temp:habitat	2	76	1,649	0,1991
Pmem	lec1	(Intercept)	1	76	124,303	<0,0001
		temp	2	76	0,720	0,4899
		habitat	1	6	7,319	0,0353
		temp:habitat	2	76	1,723	0,1855
Pmem	swi10	(Intercept)	1	76	894,075	<0,0001

		temp	2	76	24,549	<0,0001
		habitat	1	6	0,649	0,4512
		temp:habitat	2	76	0,037	0,9640
Pmem	spp	(Intercept)	1	76	196,728	<0,0001
		temp	2	76	19,404	<0,0001
		habitat	1	6	0,581	0,4749
		temp:habitat	2	76	0,022	0,9782
Pmem	hhk2	(Intercept)	1	76	790,794	<0,0001
		temp	2	76	70,899	<0,0001
		habitat	1	6	0,554	0,4847
		temp:habitat	2	76	0,980	0,3800
Pmem	mot1	(Intercept)	1	76	5,044	0,0276
		temp	2	76	103,617	<0,0001
		habitat	1	6	0,200	0,6704
		temp:habitat	2	76	1,566	0,2155
Pmem	msn2	(Intercept)	1	76	202,094	<0,0001
		temp	2	76	20,117	<0,0001
		habitat	1	6	0,299	0,6044
		temp:habitat	2	76	0,955	0,3893
Pmem	UCRNP2_806	(Intercept)	1	76	883,319	<0,0001
		temp	2	76	18,312	<0,0001
		habitat	1	6	0,302	0,6022
		temp:habitat	2	76	0,631	0,5349
Pmem	hsp98	(Intercept)	1	76	61,686	<0,0001
		temp	2	76	150,526	<0,0001
		habitat	1	6	0,120	0,7411
		temp:habitat	2	76	2,287	0,1085
Pmem	hsp88	(Intercept)	1	76	150,685	<0,0001
		temp	2	76	106,372	<0,0001
		habitat	1	6	0,361	0,5699
		temp:habitat	2	76	2,617	0,0796
Nostoc	hrcA	(Intercept)	1	76	1.642,662	<0,0001
		temp	2	76	141,077	<0,0001
		habitat	1	6	7,488	0,0339
		temp:habitat	2	76	0,251	0,7788
Nostoc	Npun_F4482	(Intercept)	1	76	3.177,738	<0,0001
		temp	2	76	30,561	<0,0001
		habitat	1	6	17,285	0,0060
		temp:habitat	2	76	0,716	0,4919
Nostoc	hsp90	(Intercept)	1	76	2.016,125	<0,0001
		temp	2	76	34,713	<0,0001
		habitat	1	6	0,861	0,3894
		temp:habitat	2	76	0,993	0,3752
Nostoc	dnaJ	(Intercept)	1	76	622,427	<0,0001
		temp	2	76	99,262	<0,0001

		habitat	1	6	0,026	0,8768
		temp:habitat	2	76	2,843	0,0645
Nostoc	groEL	(Intercept)	1	76	66,906	<0,0001
		temp	2	76	12,521	<0,0001
		habitat	1	6	0,541	0,4896
		temp:habitat	2	76	1,318	0,2738
Nostoc	recO	(Intercept)	1	76	1.193,277	<0,0001
		temp	2	76	24,745	<0,0001
		habitat	1	6	0,029	0,8699
		temp:habitat	2	76	0,560	0,5734
Nostoc	recN	(Intercept)	1	76	2.931,492	<0,0001
		temp	2	76	42,159	<0,0001
		habitat	1	6	2,785	0,1462
		temp:habitat	2	76	0,292	0,7478
Nostoc	recF	(Intercept)	1	76	3.656,231	<0,0001
		temp	2	76	179,418	<0,0001
		habitat	1	6	2,482	0,1662
		temp:habitat	2	76	0,326	0,7230
Nostoc	radA	(Intercept)	1	76	1.193,193	<0,0001
		temp	2	76	70,071	<0,0001
		habitat	1	6	0,006	0,9404
		temp:habitat	2	76	0,400	0,6719

Table S7: Detailed ANOVA output for linear model with „sea distance“ and „temperature“ as factors. Interaction effects only shown when significant.

Species	**Gene**	**Factor**	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
Pmem	Lec2	(Intercept)	1,124330	0,541740	2,0750	0,0401
		distance	0,053280	0,010040	5,3090	<0,0001
		temp	0,173390	0,029460	5,8860	<0,0001
Pmem	Lec1	(Intercept)	3,927428	0,458437	8,5670	<0,0001
		distance	0,039690	0,008493	4,6730	<0,0001
		temp	-0,015937	0,024930	-0,6390	0,5240
Pmem	swi10	(Intercept)	6,313661	0,143569	43,9760	<0,0001
		distance	0,015877	0,002660	5,9700	<0,0001
		temp	-0,043598	0,007807	-5,5840	<0,0001
Pmem	spp	(Intercept)	4,984109	0,207987	23,9640	<0,0001
		distance	0,026887	0,003853	6,9780	<0,0001
		temp	-0,046966	0,011311	-4,1520	<0,0001
Pmem	hhk2	(Intercept)	4,329989	0,202419	21,3910	<0,0001
		distance	0,009558	0,003750	2,5490	0,0121
		temp	-0,052389	0,011008	-4,7590	<0,0001
Pmem	mot1	(Intercept)	4,935902	0,202085	24,4250	<0,0001
		distance	0,023339	0,003744	6,2340	<0,0001
		temp	-0,044948	0,010990	-4,0900	<0,0001

Pmem	msn2	(Intercept)	0,338178	0,210713	1,6050	0,1110
		distance	0,018186	0,003903	4,6590	<0,0001
		temp	-0,088601	0,011459	-7,7320	<0,0001
Pmem	UCRNP2_806	(Intercept)	6,925021	0,178676	38,7570	<0,0001
		distance	0,015333	0,003310	4,6330	<0,0001
		temp	-0,049950	0,009717	-5,1410	<0,0001
Pmem	hsp98	(Intercept)	5,185239	0,270914	19,1400	<0,0001
		distance	0,024667	0,005019	4,9150	<0,0001
		temp	-0,103411	0,014733	-7,0190	<0,0001
Pmem	hsp88	(Intercept)	3,513851	0,225884	15,5560	<0,0001
		distance	0,012393	0,004185	2,9620	0,00371
		temp	-0,099797	0,012284	-8,1240	<0,0001
Nostoc	hrcA	(Intercept)	6,290411	0,167869	37,4720	<0,0001
		distance	-0,008721	0,005376	-1,6220	0,1075
		temp	-0,069880	0,009829	-7,1090	<0,0001
		distance:temp	0,000758	0,000315	2,4070	0,0176
Nostoc	Npun_F4482	(Intercept)	7,091614	0,123474	57,4340	<0,0001
		distance	-0,019411	0,002287	-8,4860	<0,0001
		temp	-0,036585	0,006715	-5,4490	<0,0001
Nostoc	hsp90	(Intercept)	6,723761	0,199557	33,6900	<0,0001
		distance	0,018113	0,003697	4,9000	<0,0001
		temp	-0,108631	0,010852	-10,0100	<0,0001
Nostoc	dnaJ	(Intercept)	4,457423	0,135070	33,0010	<0,0001
		distance	-0,004925	0,002502	-1,9680	0,0514
		temp	-0,068294	0,007345	-9,2980	<0,0001
Nostoc	groEL	(Intercept)	1,729570	0,304980	5,6710	<0,0001
		distance	-0,004320	0,005650	-0,7650	0,44603
		temp	0,053440	0,016580	3,2220	0,00165
Nostoc	recO	(Intercept)	3,803037	0,171892	22,1250	<0,0001
		distance	0,003759	0,003184	1,1800	0,2400
		temp	0,074287	0,009348	7,9470	<0,0001
Nostoc	recN	(Intercept)	5,232463	0,138133	37,8800	<0,0001
		distance	0,000655	0,002559	0,2560	0,798
		temp	0,035761	0,007512	4,7610	<0,0001
Nostoc	recF	(Intercept)	6,567106	0,198908	33,0160	<0,0001
		distance	0,004549	0,003685	1,2340	0,2200
		temp	0,074737	0,010817	6,9090	<0,0001
Nostoc	radA	(Intercept)	5,537697	0,144905	38,2160	<0,0001
		distance	0,011612	0,002684	4,3260	<0,0001
		temp	0,132383	0,007880	16,8000	<0,0001

Table S8: Detailed ANOVA output for linear model with „elevation“ and „temperature“ as factors. Interaction effects only shown when significant.

Species	**Gene**	**Factor**	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**

Pmem	Lec2	(Intercept)	1,056311	0,551027	1,9170	0,0577
		elevation	0,008810	0,001702	5,1760	<0,0001
		temp	0,173392	0,029603	5,8570	<0,0001
Pmem	Lec1	(Intercept)	3,479040	0,414839	8,3860	<0,0001
		elevation	0,009652	0,001281	7,5320	<0,0001
		temp	-0,015937	0,022287	-0,7150	0,4760
Pmem	swi10	(Intercept)	6,540402	0,164635	39,7270	<0,0001
		elevation	0,000707	0,000509	1,3900	0,1670
		temp	-0,043598	0,008845	-4,9290	<0,0001
Pmem	spp	(Intercept)	5,305027	0,245404	21,6180	<0,0001
		elevation	0,001687	0,000758	2,2250	0,02800
		temp	-0,046966	0,013184	-3,5620	0,00053
Pmem	hhk2	(Intercept)	4,518000	0,210500	21,4620	<0,0001
		elevation	0,000026	0,000650	0,0400	0,9680
		temp	-0,052390	0,011310	-4,6320	<0,0001
Pmem	mot1	(Intercept)	5,208469	0,231733	22,4760	<0,0001
		elevation	0,001511	0,000716	2,1110	0,0369
		temp	-0,044948	0,012450	-3,6100	0,0005
Pmem	msn2	(Intercept)	0,554617	0,229689	2,4150	0,0173
		elevation	0,001146	0,000710	1,6150	0,1091
		temp	-0,088602	0,012340	-7,1800	<0,0001
Pmem	UCRNP2_806	(Intercept)	7,263355	0,196615	36,9420	<0,0001
		elevation	-0,000244	0,000607	-0,4020	0,6880
		temp	-0,049951	0,010563	-4,7290	<0,0001
Pmem	hsp98	(Intercept)	5678,000	301,2000	18,8500	<0,0001
		elevation	0,007256	0,930400	0,0080	0,9940
		temp	-103,4000	16,18000	-6,3900	<0,0001
Pmem	hsp88	(Intercept)	3,823772	0,236626	16,1600	<0,0001
		elevation	-0,000481	0,000731	-0,6580	0,5120
		temp	-0,099797	0,012713	-7,8500	<0,0001
Nostoc	hrcA	(Intercept)	5,957798	0,139145	42,8170	<0,0001
		elevation	0,001228	0,000430	2,8570	0,0051
		temp	-0,054713	0,007475	-7,3190	<0,0001
Nostoc	Npun_F4482	(Intercept)	7,010516	0,141884	49,4100	<0,0001
		elevation	-0,002387	0,000438	-5,4470	<0,0001
		temp	-0,036585	0,007623	-4,8000	<0,0001
Nostoc	hsp90	(Intercept)	6,816681	0,212718	32,0460	<0,0001
		elevation	0,002094	0,000657	3,1860	0,0019
		temp	-0,108631	0,011428	-9,5060	<0,0001
Nostoc	dnaJ	(Intercept)	4,270882	0,137455	31,0710	<0,0001
		elevation	0,000683	0,000425	1,6090	0,1100
		temp	-0,068295	0,007385	-9,2480	<0,0001
Nostoc	groEL	(Intercept)	1,424126	0,305264	4,6650	<0,0001
		elevation	0,001701	0,000943	1,8040	0,0738
		temp	0,053444	0,016400	3,2590	0,0015

Nostoc	recO	(Intercept)	3,694321	0,169726	21,7660	<0,0001
		elevation	0,001429	0,000524	2,7250	0,0074
		temp	0,074287	0,009118	8,1470	<0,0001
Nostoc	recN	(Intercept)	5,084209	0,134738	37,7340	<0,0001
		elevation	0,001253	0,000416	3,0110	0,0032
		temp	0,035761	0,007239	4,9400	<0,0001
Nostoc	recF	(Intercept)	6,490010	0,198844	32,6390	<0,0001
		elevation	0,001306	0,000614	2,1260	0,0356
		temp	0,074737	0,010683	6,9960	<0,0001
Nostoc	radA	(Intercept)	6,000000	0,148184	37,0000	<0,0001
		elevation	0,001829	0,000458	4,0000	0,0001
		temp	0,132383	0,007961	17,0000	<0,0001

Correlation analysis

Table S9: Correlation coefficients for all candidate genes from correlation analysis

	lec1	lec2	hsp90	Npun_F4482	hsp88	hsp98	UCRNP2_806	msn2	mot1	hhk2	spp	swi10	dnaJ	groEL	hrcA	radA	recF	recN	recO
lec1	1	0,2514	0,1581	-0,1333	0,1614	0,1719	0,1245	0,2302	0,2013	0,0326	0,2400	0,2497	0,0723	0,2221	0,1891	0,1729	0,1722	0,2477	0,3101
lec2	0,2514	1	-0,1380	-0,3349	-0,0871	0,0186	-0,0063	0,0990	0,0992	0,1331	0,1598	0,0581	-0,2581	0,0527	-0,1275	0,5688	0,3139	0,3333	0,5293
hsp90	0,1581	-0,1380	1	0,1993	0,3298	0,3062	0,3629	0,4116	0,3981	0,2060	0,4217	0,5222	0,3964	-0,2450	0,4912	-0,3704	-0,0869	-0,2348	-0,2373
Npun_F4482	-0,1333	-0,3349	0,1993	1	0,1365	-0,0395	-0,1316	0,0660	-0,2070	-0,0332	-0,1792	-0,0141	0,3454	0,0053	0,2731	-0,3439	-0,1002	0,0445	-0,0993
hsp88	0,1614	-0,0871	0,3298	0,1365	1	0,8719	0,5740	0,8405	0,6657	0,7066	0,6345	0,6702	0,3377	-0,3731	0,3557	-0,3323	-0,3333	-0,2043	-0,2956
hsp98	0,1719	0,0186	0,3062	-0,0395	0,8719	1	0,6371	0,7774	0,6355	0,7995	0,6802	0,7532	0,1617	-0,4005	0,1490	-0,2809	-0,4494	-0,3605	-0,2095
UCRNP2_806	0,1245	-0,0063	0,3629	-0,1316	0,5740	0,6371	1	0,6182	0,7069	0,5184	0,5642	0,6817	-0,0092	-0,3594	0,1180	-0,2204	-0,1955	-0,3534	-0,2013
msn2	0,2302	0,0990	0,4116	0,0660	0,8405	0,7774	0,6182	1	0,7403	0,6691	0,7171	0,7220	0,2486	-0,5071	0,3986	-0,2462	-0,2367	-0,1234	-0,2171
mot1	0,2013	0,0992	0,3981	-0,2070	0,6657	0,6355	0,7069	0,7403	1	0,5299	0,8695	0,7975	0,0004	-0,4481	0,1478	0,0001	-0,0111	-0,1687	-0,1461
hhk2	0,0326	0,1331	0,2060	-0,0332	0,7066	0,7995	0,5184	0,6691	0,5299	1	0,4760	0,5282	0,1721	-0,2509	0,1218	-0,2283	-0,3920	-0,3261	-0,2084
spp	0,2400	0,1598	0,4217	-0,1792	0,6345	0,6802	0,5642	0,7171	0,8695	0,4760	1	0,8086	-0,0383	-0,4784	0,0892	0,0178	-0,0953	-0,1709	-0,0269
swi10	0,2497	0,0581	0,5222	-0,0141	0,6702	0,7532	0,6817	0,7220	0,7975	0,5282	0,8086	1	0,0303	-0,4450	0,1575	-0,0913	-0,2044	-0,2627	-0,0326
dnaJ	0,0723	-0,2581	0,3964	0,3454	0,3377	0,1617	-0,0092	0,2486	0,0004	0,1721	-0,0383	0,0303	1	0,0772	0,6400	-0,4351	-0,1496	0,1440	-0,3417
groEL	0,2221	0,0527	-0,2450	0,0053	-0,3731	-0,4005	-0,3594	-0,5071	-0,4481	-0,2509	-0,4784	-0,4450	0,0772	1	-0,1778	0,2215	0,2612	0,3416	0,2551
hrcA	0,1891	-0,1275	0,4912	0,2731	0,3557	0,1490	0,1180	0,3986	0,1478	0,1218	0,0892	0,1575	0,6400	-0,1778	1	-0,2941	0,0580	0,1908	-0,1893
radA	0,1729	0,5688	-0,3704	-0,3439	-0,3323	-0,2809	-0,2204	-0,2462	0,0001	-0,2283	0,0178	-0,0913	-0,4351	0,2215	-0,2941	1	0,6542	0,5243	0,7417
recF	0,1722	0,3139	-0,0869	-0,1002	-0,3333	-0,4494	-0,1955	-0,2367	-0,0111	-0,3920	-0,0953	-0,2044	-0,1496	0,2612	0,0580	0,6542	1	0,6490	0,4437
recN	0,2477	0,3333	-0,2348	0,0445	-0,2043	-0,3605	-0,3534	-0,1234	-0,1687	-0,3261	-0,1709	-0,2627	0,1440	0,3416	0,1908	0,5243	0,6490	1	0,4615
recO	0,3101	0,5293	-0,2373	-0,0993	-0,2956	-0,2095	-0,2013	-0,2171	-0,1461	-0,2084	-0,0269	-0,0326	-0,3417	0,2551	-0,1893	0,7417	0,4437	0,4615	1

Table S10: P-values for all candidate genes from correlation analysis

	lec1	lec2	hsp90	Npun_F4482	hsp88	hsp98	UCRNP2_806	msn2	mot1	hhk2	spp	swi10	dnaJ	groEL	hrcA	radA	recF	recN	recO
lec1		0,0056	0,0845	0,1467	0,0781	0,0604	0,1753	0,0114	0,0275	0,7233	0,0083	0,0060	0,4324	0,0147	0,0385	0,0590	0,0600	0,0064	0,0006
lec2	0,0056		0,1329	0,0002	0,3443	0,8401	0,9457	0,2819	0,2811	0,1472	0,0813	0,5288	0,0044	0,5672	0,1653	0,0000	0,0005	0,0002	0,0000
hsp90	0,0845	0,1329		0,0291	0,0002	0,0007	0,0000	0,0000	0,0000	0,0240	0,0000	0,0000	0,0000	0,0070	0,0000	0,0000	0,3454	0,0098	0,0091
Npun_F4482	0,1467	0,0002	0,0291		0,1372	0,6680	0,1519	0,4738	0,0233	0,7188	0,0502	0,8787	0,0001	0,9540	0,0025	0,0001	0,2761	0,6293	0,2805
hsp88	0,0781	0,3443	0,0002	0,1372		0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0002	0,0000	0,0000	0,0002	0,0002	0,0252	0,0010
hsp98	0,0604	0,8401	0,0007	0,6680	0,0000		0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0777	0,0000	0,1043	0,0019	0,0000	0,0000	0,0217
UCRNP2_806	0,1753	0,9457	0,0000	0,1519	0,0000	0,0000		0,0000	0,0000	0,0000	0,0000	0,0000	0,9204	0,0000	0,1993	0,0156	0,0324	0,0000	0,0274
msn2	0,0114	0,2819	0,0000	0,4738	0,0000	0,0000	0,0000		0,0000	0,0000	0,0000	0,0000	0,0062	0,0000	0,0000	0,0067	0,0092	0,1794	0,0172
mot1	0,0275	0,2811	0,0000	0,0233	0,0000	0,0000	0,0000	0,0000		0,0000	0,0000	0,0000	0,9961	0,0000	0,1073	0,9995	0,9038	0,0655	0,1114
hhk2	0,7233	0,1472	0,0240	0,7188	0,0000	0,0000	0,0000	0,0000	0,0000		0,0000	0,0000	0,0602	0,0057	0,1849	0,0121	0,0000	0,0003	0,0224
spp	0,0083	0,0813	0,0000	0,0502	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000		0,0000	0,6783	0,0000	0,3324	0,8471	0,3006	0,0620	0,7706
swi10	0,0060	0,5288	0,0000	0,8787	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000		0,7422	0,0000	0,0857	0,3212	0,0252	0,0037	0,7234
dnaJ	0,4324	0,0044	0,0000	0,0001	0,0002	0,0777	0,9204	0,0062	0,9961	0,0602	0,6783	0,7422		0,4018	0,0000	0,0000	0,1030	0,1167	0,0001
groEL	0,0147	0,5672	0,0070	0,9540	0,0000	0,0000	0,0000	0,0000	0,0000	0,0057	0,0000	0,0000	0,4018		0,0520	0,0151	0,0040	0,0001	0,0049
hrcA	0,0385	0,1653	0,0000	0,0025	0,0000	0,1043	0,1993	0,0000	0,1073	0,1849	0,3324	0,0857	0,0000	0,0520		0,0011	0,5294	0,0369	0,0384
radA	0,0590	0,0000	0,0000	0,0001	0,0002	0,0019	0,0156	0,0067	0,9995	0,0121	0,8471	0,3212	0,0000	0,0151	0,0011		0,0000	0,0000	0,0000
recF	0,0600	0,0005	0,3454	0,2761	0,0002	0,0000	0,0324	0,0092	0,9038	0,0000	0,3006	0,0252	0,1030	0,0040	0,5294	0,0000		0,0000	0,0000
recN	0,0064	0,0002	0,0098	0,6293	0,0252	0,0000	0,0000	0,1794	0,0655	0,0003	0,0620	0,0037	0,1167	0,0001	0,0369	0,0000	0,0000		0,0000
recO	0,0006	0,0000	0,0091	0,2805	0,0010	0,0217	0,0274	0,0172	0,1114	0,0224	0,7706	0,7234	0,0001	0,0049	0,0384	0,0000	0,0000	0,0000	

Principal component analysis (PCA)

Table S11: Gene loadings of principle components PC1-3.

	PC1	PC2	PC3
lec1	0,30754	0,21275	-0,85287
lec2	0,86535	0,02149	0,33238
hsp90	-0,06286	0,21512	-0,09849
Npun_F4482	-0,08382	-0,00231	-0,03140
hsp88	-0,04576	0,36967	0,01605
hsp98	-0,01009	0,45124	0,07562
UCRNP2_806	-0,00775	0,22145	0,03730
msn2	0,02350	0,35931	0,03904
mot1	0,03775	0,28752	0,04157
hhk2	0,01561	0,23818	0,11343
spp	0,06100	0,30498	0,04330
swi10	0,01663	0,22002	0,00572
dnaJ	-0,06987	0,05905	-0,09161
groEL	0,06887	-0,23938	-0,30449
hrcA	-0,02988	0,08063	-0,08638
radA	0,25305	-0,12585	0,01247
recF	0,13929	-0,12027	-0,08676
recN	0,08926	-0,06906	-0,07774
recO	0,18763	-0,07994	-0,06131

Table S12: Detailed ANOVA output for PC1-3 for linear mixed effects model with „temperature“ and „site“ as fixed and „individual“ as random factor.

	factor	numDF	denDF	F-value	p-value
PC1	(Intercept)	1	92	0,0000	1,0000
	temp	2	92	42,9750	<0,0001
	site	7	92	11,7552	<0,0001
	temp:site	14	92	0,8898	0,5721
PC2	(Intercept)	1	92	0,0000	1,0000
	temp	2	92	147,1362	<0,0001
	site	7	92	28,2533	<0,0001
	temp:site	14	92	2,1405	0,0162
PC3	(Intercept)	1	92	0,0000	1,0000
	temp	2	92	307,6892	0,0509
	site	7	92	4,0572	0,0006
	temp:site	14	92	0,2406	0,9977

Table S13: Detailed Tukey test ouput for PC1-3 for linear mixed effects model with „temperature“ and „site“ as fixed and „individual“ as random factors for single temperature comparision.

	contrast	estimate	SE	df	t.ratio	p.value
PC1	5°C vs. 15°C	-3,819725	0,5217192	92	-7.321	<0,0001
	5°C vs. 25°C	-4,479564	0,5217192	92	-8.586	<0,0001
	15°C vs. 25°C	-0,659839	0,5217192	92	-1.265	0,4187
PC2	5°C vs. 15°C	0,520314	0,3258694	92	1.597	0,2522
	5°C vs. 25°C	5,080294	0,3258694	92	15.590	<0,0001
	15°C vs. 25°C	4,559980	0,3258694	92	13.993	<0,0001
PC3	5°C vs. 15°C	-1,156745	0,4791903	92	-2.414	0,0463
	5°C vs. 25°C	-0,815532	0,4791903	92	-1.702	0,2100
	15°C vs. 25°C	0,341214	0,4791903	92	0,712	0,7570

Table S14: Detailed ANOVA output for PC1-3 for linear mixed effects model with „temperature“ and „habitat“ as fixed and „individual“ and „site“ as random factors.

	factor	numDF	denDF	F-value	p-value
PC1	(Intercept)	1	76	0,0000	1,0000
	temp	2	76	69,0417	<0,0001
	habitat	1	6	11,7282	0,0141
	temp:habitat	2	76	2,4556	0,0926
PC2	(Intercept)	1	76	0,0000	1,0000
	temp	2	76	145,1601	<0,0001
	habitat	1	6	0,6109	0,4642
	temp:habitat	2	76	1,0169	0,3666
PC3	(Intercept)	1	76	0,0000	1,0000
	temp	2	76	4,9213	0,0098
	habitat	1	6	2,4486	0,1687
	temp:habitat	2	76	0,3631	0,6967

Table S15: Detailed ANOVA output for PC1-3 for linear mixed effects model with „temperature“ and „sea distance“ as fixed and „individual“ and „site“ as random factors.

	factor	numDF	denDF	F-value	p-value
PC1	(Intercept)	1	76	0,0000	1,0000
	temp	2	76	69,4927	<0,0001
	distance	1	6	11,8096	0,0139
	temp:distance	2	76	2,7198	0,0723
PC2	(Intercept)	1	76	0,0000	1,0000
	temp	2	76	149,8111	<0,0001
	distance	1	6	3,2712	0,1205
	temp:distance	2	76	2,2670	0,1106
PC3	(Intercept)	1	76	0,0000	1,0000
	temp	2	76	4,9291	0,0097

	distance	1	6	0,5540	0,4848
	temp:distance	2	76	0,4235	0,6563

Table S16: Detailed ANOVA output for PC1-3 for linear mixed effects model with „temperature“ and „elevation“ as fixed and „individual“ as random factor.

	factor	**numDF**	**denDF**	**F-value**	**p-value**
PC1	(Intercept)	1	76	0,0000	1,0000
	temp	2	76	72,2156	<0,0001
	elevation	1	6	39,7260	0,0007
	temp:elevation	2	76	4,3153	0,0168
PC2	(Intercept)	1	76	0,0000	1,0000
	temp	2	76	149,9104	<0,0001
	elevation	1	6	0,5445	0,4884
	temp:elevation	2	76	2,2941	0,1078
PC3	(Intercept)	1	76	0,0000	1,0000
	temp	2	76	4,9156	0,0098
	elevation	1	6	10,1785	0,0188
	temp:elevation	2	76	0,3182	0,7285