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Genome-wide transcriptomic responses of the seagrasses *Zostera marina* and *Nanozostera noltii* under a simulated heatwave confirm functional types

Susanne U. Franssen [a,*,1], Jenny Gu [a,1], Gidon Winters [a], Ann-Kathrin Huylmans [a], Isabell Wienpahl [a], Maximiliane Sparwel [a], James A. Coyer [b,2], Jeanine L. Olsen [b], Thorsten B.H. Reusch [c], Erich Bornberg-Bauer [a]

[a] Institute for Evolution and Biodiversity, University of Münster, Hüfferstr. 1, 48149 Münster, Germany
[b] Marine Benthic Ecology and Evolution Group, Centre for Ecological and Evolutionary Studies, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands
[c] GEOMAR Helmholtz-Centre for Ocean Research Kiel, Evolutionary Ecology of Marine Fishes, Düsternbrooker Weg 20, 24105 Kiel, Germany

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ABSTRACT

Genome-wide transcription analysis between related species occurring in overlapping ranges can provide insights into the molecular basis underlying different ecological niches. The co-occurring seagrass species, *Zostera marina* and *Nanozostera noltii*, are found in marine coastal environments throughout the northern hemisphere. *Z. marina* is often dominant in subtidal environments and subjected to fewer temperature extremes compared to the predominately intertidal and more stress-tolerant *N. noltii*.

We exposed plants of both species to a realistic heat wave scenario in a common-stress-garden experiment. Using RNA-seq (~7 million reads/library), four *Z. marina* and four *N. noltii* libraries were compared representing northern (Denmark) and southern (Italy) locations within the co-occurring range of the species' European distribution.

A total of 8977 expressed genes were identified, of which 78 were directly related to heat stress. As predicted, both species were negatively affected by the heat wave, but showed markedly different molecular responses. In *Z. marina* the heat response was similar across locations in response to the heatwave at 26 °C, with a complex response in functions related to protein folding, synthesis of ribosomal chloroplast proteins, proteins involved in cell wall modification and heat shock proteins (HSPs). In *N. noltii* the heat response markedly differed between locations, while HSP genes were not induced in either population.

Our results suggest that as coastal seawater temperatures increase, *Z. marina* will disappear along its southern most ranges, whereas *N. noltii* will continue to move north. As a consequence, sub- and intertidal habitat partitioning may weaken in more northern regions because the higher thermal tolerance of *N. noltii* provides a competitive advantage in both habitats. Although previous studies have focused on HSPs, the present study clearly demonstrates that a broader examination of stress related genes is necessary.

1. Introduction

Species-specific patterns of gene expression are predicted to correlate with their ecological niches and can now be compared and analyzed using global transcription analysis via RNA-seq. For example, inter-species transcriptomics of the invasive cordgrass *Spartina alterniflora* and the native *Spartina maritima*, suggested that the competitive success of the invasive congener might be due to growth advantages and a higher stress tolerance (Chelaifa et al., 2010). In the present study, we apply inter-species transcriptomics to two, closely related marine flowering plants that occupy different ecological niches (Den Hartog, 1970; Phillips and Menez, 1988).

The seagrasses, *Zostera marina* (eelgrass) and *Nanozostera noltii* (dwarf eelgrass; formerly *Zostera noltii*) (Coyer et al., 2013) diverged ~14 Mya (Kato et al., 2003; Coyer et al., 2013). They provide the foundational habitat for the seagrass community of many, soft-sediment, coastal systems along European coasts. *Z. marina* ranges from southern Portugal to northern Norway and Iceland, as well as into warm temperate areas of the Mediterranean, where it becomes more sparse (Borum et al., 2004). In contrast, *N. noltii* ranges from southern Norway to Mauritania, also including the Mediterranean, Black, Aral, and Caspian

* Corresponding author at: Institut für Populationsgenetik, Vetmeduni Vienna, Veterinärplatz 1, 1210 Vienna, Austria.
E-mail address: susanne.franssen@vetmeduni.ac.at (S.U. Franssen).
[1] Shared first authorship.
[2] Present address: Shoals Marine Laboratory, Cornell University, 400 Little Harbor Road, Portsmouth, NH 03801, USA.

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Seas (Phillips and Menez, 1988; Borum et al., 2004). The two species overlap in their distributional range roughly between the northern Mediterranean and southern Norway.

Z. marina is predominantly subtidal, particularly in warmer southern European locations (Laugier et al., 1999; Billingham et al., 2003; Massa et al., 2008), where it experiences relatively constant physical conditions and fewer extreme temperatures due to the balancing effect of the surrounding water column. In more northerly latitudes it occurs both subtidally (northern Denmark) and, to a lesser extent, intertidally, (Wadden Sea) (Oetjen and Reusch, 2007). At the Thau Lagoon location (Mediterranean coast of France) it is sheltered, permanently supplied with nutrients and less exposed to environmental extremes (Laugier et al., 1999). In contrast, *N. noltii* is predominantly an intertidal species, where it experiences more variable environmental conditions of sea and air exposure, as well as physical stressors such as wind and waves (Laugier et al., 1999; Massa et al., 2008). In the Ria Formosa Lagoon in southern Portugal, *N. noltii* experiences summer temperatures of 36 °C during tidal exposure, which is mainly a function of air temperatures and irradiance due to the thin water columns characteristic of intertidal pools (Massa et al., 2008, 2011). In this environment local extinction of *Z. marina* has been correlated with the warmest summers in the Ria Formosa from 2003 to 2008 (Massa et al., 2008).

Extreme weather events are increasing under global warming scenarios and are predicted to have strong influences on ecosystems and associated species (Easterling et al., 2000; Walther et al., 2002). Water temperatures of ~25 °C are the critical threshold for *Z. marina* in northern Europe (Reusch et al., 2005; Nejrup and Pedersen, 2008; Bergmann et al., 2010), but not for *N. noltii*. Thus, the northerly range expansion of *N. noltii* may well blur subtidal–intertidal niche dynamics. Interest in niche comparisons, as well as shifting biogeographic ranges, are therefore relevant to understanding the effects of climate change.

Differential utilization of habitat by the two related species enables us to test for concomitant differences in the molecular mechanisms that respond to a variety of stressors, particularly thermal stress. An important expression response to thermal stress is up-regulation of genes encoding heat-shock proteins (HSPs). HSPs promote cellular thermal tolerance through a variety of mechanisms, including protein folding or chaperoning of existing and newly synthesized proteins, aggregation suppression, reactivation of denatured proteins, shuttling proteins between different cell compartments, and destruction of damaged proteins (Vierling, 1991; Wahid et al., 2007; Kotak et al., 2007). Though HSP induction is a universal response to heat-stress (Vierling, 1991), species from different climatic zones show different HSP induction thresholds (Feder and Hofmann, 1999). Some of the most extreme examples come from Antarctic algae that induce HSPs at 5 °C (Vayda and Yuan, 1994), while hyperthermophilic Archaea require temperatures of 100 °C for HSP induction (Feder and Hofmann, 1999). In addition, the correlation between habitat temperatures and HSP induction thresholds has been observed for congeners from habitats with much more subtle temperature differences (Ulmasov et al., 1992; Feder and Hofmann, 1999; Knight, 2010).

Heat-stress tolerance is, however, a multigenic trait and non-HSP genes are also essential (Larkindale et al., 2005; Wahid et al., 2007; Kotak et al., 2007). These include expression changes to allow the maintenance of membrane stability, scavenging of reactive oxygen species, production of antioxidants, accumulation and adjustment of compatible osmolytes and induction of signaling cascades (Wahid et al., 2007; Kotak et al., 2007). It has further been suggested that the acute stress response and the long term adaptation to stress are based on separate mechanisms and that HSP expression does not necessarily play a major role for the evolutionary adaptation to higher temperatures (Sørensen et al., 2007).

In this study we use RNA-seq to investigate the inter-specific transcriptomic response of *Z. marina* and *N. noltii* under a simulated heat wave based on actual conditions that occurred in the southwestern Baltic Sea in 2003, in which *Z. marina* populations were decimated (Reusch et al., 2005). Expression profiles were investigated in a common-stress-garden design using plants from northern and southern European localities, where the species co-occur. Specifically, we: 1) identify putative genes and molecular functions involved in the stress response, 2) quantify differences in the transcriptomic response of both species relevant to their respective ecological niches, 3) identify potential mechanisms of microevolutionary adaptation towards increased temperatures, and 4) discuss potential impacts of global warming on the species' distribution.

2. Material & methods

2.1. Study species and experimental design

Full details of the experimental setup are outlined in Gu et al. (2012). Briefly, individuals of *Z. marina* and *N. noltii* were collected in the spring 2009 from a northern European location (western Baltic/Kattegat, Hals, Denmark; 56°50′ N, 10°1′ E, 2009, hereafter “northern populations”) and a southern European location (Adriatic Sea; Gabicce Mare, Italy; 43°50′ N, 12°45′ E, late April, hereafter “southern populations”). At both locations, both species co-occur in the intertidal to the shallow subtidal. Summer surface water temperatures ranged from 13 °C to 22 °C (mean 18 °C) in the northern location and 21 °C to 29 °C (mean 25 °C) in the southern location based on in situ records covering the previous six years (Fig. S1).

Within each population, ca. 30 shoots (leaf bundles plus attached rhizome) were harvested from each of 15 sub-plots (total 450 shoots), which were separated by 10 m, to minimize the chance of collecting shoots from the same genotype (i.e., clone) (Bergmann et al., 2010). Shoots were transported in coolers filled with seawater and planted in the AQUATRON (a mesocosm facility installed at the University of Münster, Germany) within 48 h of collection. The experimental design is shown in Fig. S2. As described in Gu et al. (2012), the AQUATRON consisted of two temperature-controlled semi-connected water circuits, each with six 700-L mesocosms and a storage tank. All mesocosms contained artificial seawater adjusted to 28 psu (practical salinity units: 1 psu ~ 0.1% salinity) and illuminated under light-saturating conditions (~400 μmol photons s^{-1} m^{-2}). Shoots were planted into boxes with a sediment height of 10 cm (details see Fig. S2). Two boxes for each of all four populations were placed into each mesocosm (=6 independent replicate units per population and treatment condition) (Fig. S2). All shoots were genotyped with four microsatellite loci for *Z. marina* and five for *N. noltii* to verify that all genotypes were unique (Reusch, 2000; Coyer et al., 2004).

2.2. Heat wave simulation

Plants were acclimatized for 50 days, during which the water temperature in all mesocosms was slowly raised from 14 °C (collection temperature) to 19 °C (experimental control temperature) (Fig. S3). Following acclimation, a heat wave was initiated in a common-stress-garden approach. Six experimental units were maintained at the control temperature of 19 °C, while the temperature in the remaining six was gradually increased by 1 °C per day, up to 26 °C, and held for 3 weeks; then decreased by 1 °C per day to the control temperature of 19 °C (Fig. S3). The experimental profile mirrored the temperature profile observed during a heat wave in summer 2003 in the shallow waters of the western Baltic Sea (Reusch et al., 2005).

2.3. Evaluation of plant performance

Plant performance was estimated by changes in shoot number from the start of the experiment until the midpoint of the heat wave and ca. 1.5 weeks after the end of the heat wave (Fig. S3). Changes in shoot number from the beginning of the heat wave to the respective time point were normalized to the starting number of shoots in the respective box. Normalized changes were fitted to a generalized linear

model with the additive factors treatment and population, and statistical significance of both factors was tested.

2.4. RNA extraction, library preparation and sequencing

We used RNA samples described in Gu et al. (2012). Briefly, RNA was sampled by cutting young and epiphyte-free leaf tips from the second leaf of *Z. marina* (4 cm) and *N. noltii* (10 cm), then immediately frozen in liquid nitrogen. Frozen tissue was pulverized with a Retsch Mixer Mill MM301 (Qiagen) and RNA extracted with the Invisorb RNA plant HTS 96 extraction kit (Invitek). For comparative expression analysis, eight treatments (*Zm*, north, control; *Zm*, north, heat; *Zm*, south, control; *Zm*, south, heat; repeated for *Nn*) were sampled at the mid-point of the heat wave (Fig. S3). For each RNA-seq library, RNA was pooled from seven different genotypes of the respective experimental condition. Total RNA (ca. ~5 μg per library) was sheared with ultrasound and 3′ polyA fragments were purified by oligo(dT) chromatography (3′ UTR isolation). First-strand cDNA synthesis was performed using oligo(dT) priming followed by 12–15 cycles of PCR (GATC Biotech, Konstanz, Germany; proprietary protocol). Resulting cDNA libraries were tagged and sequenced in four lanes (2 libraries per lane) with the Illumina Genome Analyzer II (read length 76 bp).

Gu et al. (2012) used a subset of the libraries used here. In their study, changes in metabolite composition were related to the transcriptomic response involved in metabolic processes obtained from the RNA-seq reads of the Illumina libraries and annotated from the Metacyc data base (≈35% of the total annotated genes used here) (Caspi et al., 2008; Gu et al., 2012). The current study extends the previous work by including the complete transcriptomic response, accounting for biological variation in a differential expression analysis framework (see Section 2.6–2.8) and the focus on ecological differences of both species.

2.5. Generation of expression profiles

No genomic reference exists for either seagrass species, thus a transcriptomic reference was used for read mapping using BWA v0.5.8 (Li and Durbin, 2009) of the reads primed in the 3′ UTR from the eight RNA-seq libraries. For *Z. marina*, a de novo transcriptome containing 30% of all genes of a typical flowering plant (12,380 *Arabidopsis thaliana*, 12.686 *Oryza sativa* orthologs) was used as a reference (http://drzompo.uni-muenster.de/downloads; library: Zoma_C) (Wissler et al., 2009; Franssen et al., 2011a). For *N. noltii*, a de novo transcriptome described in Gu et al. (2012) using plant material from the northern and southern population was used (available at http://drzompo.uni-muenster.de/downloads, library: Nano_A; further details in the supplemental material). Gene expression profiles were obtained from second stage mapping of the 3′ UTR reads (Illumina) against the reference transcriptome and subsequent contig annotation by orthologous genes of *A. thaliana* (TAIR9, Swarbreck et al., 2008) and *O. sativa* (Rice Genome Annotation Project v6.1, Ouyang et al., 2007) via BLASTX (for a complete workflow see Fig. S4).

2.6. Multivariate analysis of expression profiles

Gene-expression profiles were analyzed by multivariate analysis to identify similarities and differences of the entire transcriptomic response between species and treatment conditions. Transcription profiles of the eight libraries were normalized for library size and composition of expressed transcripts (Robinson and Oshlack, 2010). Groupings of expression profiles based on the biological coefficient of variation between library pairs were identified with multidimensional-scaling (MDS) using the R package "edgeR" v2.5.1 (Robinson et al., 2010). Identified groupings were tested by ANOSIM analysis (analysis of similarity, tests distances within vs. between groups) implemented in the R package "vegan" v2.0–3 (Oksanen et al., 2012). Multivariate analysis and subsequent expression analysis along with plotting functions were performed in R (R Development Core Team, 2008).

2.7. Differential gene expression

Differential expression analysis was performed with the R package "edgeR", which employs an overdispersed Poisson model (negative binomial) to account for technical and biological variability, with the generalized linear model (GLM) functionality for multifactor experiments (Robinson et al., 2010; McCarthy et al., 2012). Differentially expressed genes were determined for three data sets: 1) eight libraries including samples of both species, 2) four libraries of *Z. marina* and 3) four libraries of *N. noltii*. In all three data sets, the expression profiles were normalized for library size and composition of expressed transcripts (Robinson and Oshlack, 2010). For the data set including both species (data set 1), the single factor species was fitted to the GLM to test for differential expression between both species consistent across treatments. In this case, all four libraries per species from the two different populations and treatments were used as biological replicates on the species level. For *Z. marina* alone (data set 2) the data were analyzed with GLM including the factors treatment and population (the factor population was suggested by the grouping of expression profiles; Fig. 1). Differential expression, with respect to heat treatment, was tested, while adjusting for the remaining factor. For *N. noltii* alone (data set 3) the factor "group identity" with three factor levels identified by MDS (Fig. 1) was fitted to the GLM. Genes displaying differential expression between heat and control treatment in the northern population (two of the three groups, Fig. 1) were identified. In all three data sets, the biological replication as defined by the design of the respective GLM was used to calculate the tagwise dispersion, the overdispersion value in the negative binomial model (Robinson et al., 2010; McCarthy et al., 2012). Results were corrected for multiple testing via false discovery rate (FDR) and reported with a significance threshold of FDR, $\alpha < 0.05$ (for a complete workflow see Fig. S4).

2.8. Functional enrichment analysis

Gene sets of the differentially expressed genes, between defined groups of libraries, were tested for enrichment of functional categories. All genes were annotated with the functional categories defined by MapMan (Usadel et al., 2009) via their ortholog annotation to *A. thaliana* (annotation version: Ath_AGI_TAIR9). Functional enrichment in gene sets vs. all genes was tested via Fisher's exact test and corrected for multiple testing with the false discovery rate (FDR) implemented in the software PageMan (Usadel et al., 2006).

3. Results

3.1. Transcriptome assembly & expressed genes

The ortholog mapping of the assembled contigs for *Z. marina* and *N. noltii* against the plant proteomes of *A. thaliana* and *O. sativa* revealed signs of redundancy/fragmentation between assembled contigs (Table S1A) (Franssen et al., 2011a; Gu et al., 2012), a characteristic also observed in other de novo transcriptome assemblies (Schwartz et al., 2010; Franssen et al., 2011b; Feldmeyer et al., 2011; Mundry et al., 2012). Therefore, gene identification for the subsequent expression analysis was based on orthology to *A. thaliana*. *A. thaliana* was chosen over *O. sativa* (despite the latter being a monocotyledon) as it is the better annotated plant species and the ortholog annotation of the assembled transcriptome with both references had a similar annotation success.

Importantly, verification has been shown between quantitative real time PCR analyses of 18 candidate genes and the RNA-seq results for *Z. marina*, based on the *A. thaliana* orthology (Franssen et al., 2011a). Using the orthology approach, 11,378 genes were expressed in *Z. marina* and 10,856 in *N. noltii*, with 8977 orthologous genes expressed

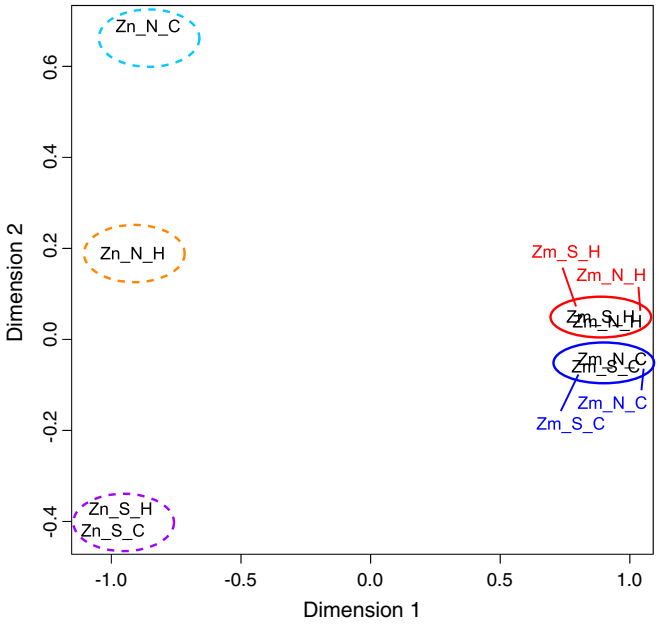

Fig. 1. Multivariate grouping of the expression profiles of the eight RNA-seq libraries. Grouping of the 8977 genes using MDS (multidimensional scaling) was based on the pairwise distances between the libraries using the biological coefficient of variation (Robinson et al., 2010). Only the most variable 25% of the genes were used for MDS analysis. Species: *Z. marina* (Zm), *N. noltii* (Nn); populations: northern (N), southern (S); heat treatment (H), control treatment (C). Groupings are indicated by color (red: Zm_N_H, Zm_S_H; blue: Zm_N_C, Zm_S_C); dashed line: *N. noltii*; solid line: *Z. marina*.

in both species. Subsequent analysis utilized the expression profiles of the 8977 genes for the eight experimental conditions (*Z. marina*/*N. noltii* ∗ north/south ∗ control/heat stress) sequenced by additional 3′ UTR Illumina sequencing with an average library size of ~7 million reads (Table S1B; for a complete workflow see Fig. S4).

3.2. Multivariate analysis of expression profiles

We compared the expression profiles using multidimensional scaling (MDS). The greatest difference was found between species (Fig. 1). In addition, five different groups of expression profiles were supported by an analysis of similarity (ANOSIM) ($R = 0.9733$; $P = 0.0025$) based on the biological coefficient of variation of the 25% most variable genes. These groupings suggested a smaller variation within expression profiles of *Z. marina* relative to *N. noltii*. For *Z. marina*, the present grouping of treatments into control and heat-stressed gene expression revealed a similar response to heat stress in both northern and southern populations. In contrast, expression profiles of *N. noltii* were more diverse between northern and southern populations. While the expression difference in response to the heat treatment was very strong in the northern population, the southern population showed a weak response with both treatments clustered in the same group. Differential expression analysis identified 59% (# 5304) differentially expressed genes between the species, with 28% (# 2524) more highly expressed in *Z. marina* and 31% (# 2780) more highly expressed in *N. noltii* (FDR $\alpha < 0.05$).

3.3. Molecular heat response: Z. marina

The similarity of expression responses to heat treatment between northern and southern populations of *Z. marina* was investigated in more detail via differential expression analysis of concordant treatment effects. In this case, treatment effects were tested disregarding population identity, i.e., RNA-seq libraries for the two populations served as biological replication. A total of 427 genes show concordant differential expression in response to the treatment with 267 up-regulated and 159 down-regulated genes under heat stress conditions (FDR $\alpha < 0.05$) (Table S2; see workflow Fig. S4).

Consistently up-regulated genes under heat-stress included several enriched functional categories. These were: 1) pectinesterases, involved in cell wall modification and subsequent breakdown of the cell wall; 2) proteins involved in the synthesis of ribosomal chloroplast proteins; and 3) proteins involved in protein folding, which contain immunophilins (endogenous cytosolic peptidyl–prolyl isomerases that interconvert between the cis and trans positions) and molecular chaperones (Fig. 2). Although the functional category "stress.abiotic.heat" was not significantly enriched, 6 genes with this term were present (Table S2) and the term "stress.abiotic" revealed a weak enrichment (Fig. 2). No HSPs were up-regulated under control conditions, instead a gene with the functional annotation "stress.abiotic.cold", a calcium-dependent lipid-binding family protein, was up-regulated (Table S2). Enriched functional categories in the gene set of up-regulated genes under control temperature were functions involved in secondary metabolism, particularly lignin biosynthesis (Fig. 2).

As some differences were observed in the heat responses of northern and southern populations of *Z. marina*, we tested the hypothesis that southern populations, originating from a warmer local climate, show stronger up-regulation of heat responsive (HR) genes than northern populations. The expression strength of the 267 up-regulated genes in response to heat in *Z. marina* showed higher expression in the southern population in comparison to the northern population in the control treatment (paired Wilcoxon test, one sided: $V = 23{,}792$, p-value $= 1.942e^{-07}$), as well as the heat treatment (paired Wilcoxon test, one sided: $V = 33{,}904$, p-value $< 2.2e^{-16}$) (Fig. 3, S5). Additionally, this directional population effect on the gene expression strength was more pronounced in the heat treatment compared to the control treatment (paired Wilcoxon test, one sided: $W = 34{,}248$, p-value $< 2.2e^{-16}$). Thus, genes that are important during heat stress showed a slight increase in their constitutive expression (under control conditions) and an even stronger increase under heat conditions (acute heat response) in the southern population as compared with the northern population.

3.4. Molecular heat response: N. noltii

Expression responses to the heat treatment in *N. noltii* were very different between the northern and southern population with a very weak response in the southern and a strong response in the northern population (Fig. 1). We further investigated genes responsible for the divergent expression in the northern population. Because no biological replication was available, we modeled the biological variation in

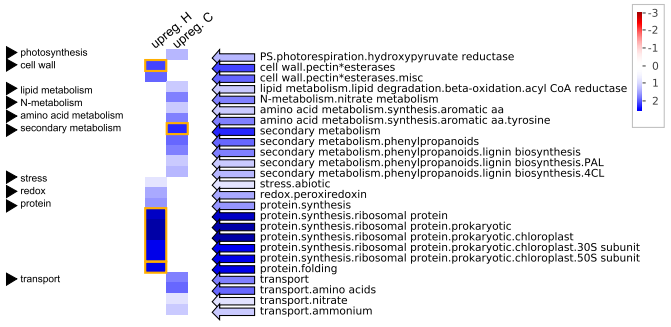

Fig. 2. Functional enrichment of differentially expressed genes in *Z. marina* populations in response to heat. Functional categories are defined via MapMan (Usadel et al., 2009). Enrichment (blue)/depletion (red) of functional categories up-regulated in either treatment condition. H: heat treatment; C: control treatment. Coloring represents z-scores of FDR corrected p-values of the enrichment test. Boxes framed in orange exceed the significance threshold of FDR $\alpha < 0.05$.

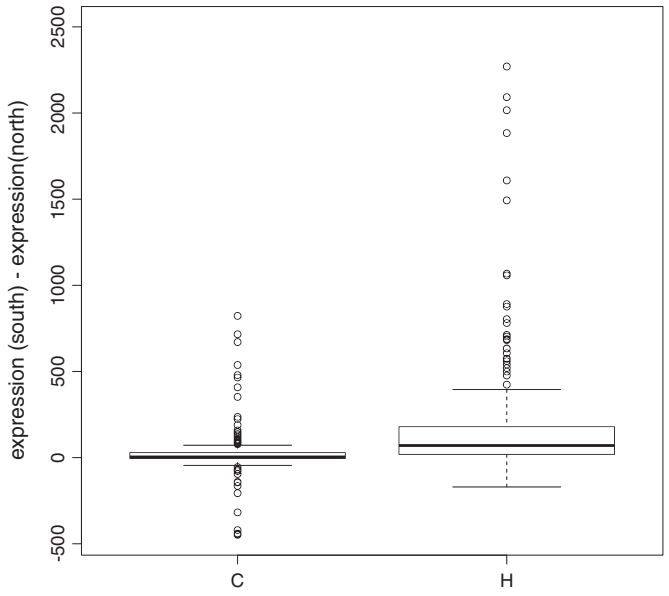

Fig. 3. Expression differences between the northern and southern population of 267 genes that were up-regulated in response to heat in *Z. marina*. Y-axis: difference in expression strength of normalized expression values between northern and southern populations of *Z. marina*. Positive values indicate higher expression in the southern population. X-axis: control treatment (C), heat treatment (H). (C: exclusion of the greatest value [all values: min. −448, 1st quantile −4, median 5, 3rd quantile 29, max. 26,620], H: exclusion of the 3 greatest values [all values: min. −170, 1st quantile 19, median 70, 3rd quantile 179, max. 134,400] in the figure due to greatly enlarging the printing range of the y-axis.).

response to heat for *N. noltii* via the biological variation between treatments of the southern population. Investigation of the strong northern response revealed differential expression of 369 genes between treatments with 28 genes up-regulated and 341 genes down-regulated upon heat treatment (see Section 2.7 "Differential gene expression"; workflow: Fig. S4; Table S2).

The up-regulated set of genes in the northern population consisted of only 28 genes, none of which encoded an HSP gene or were enriched in any functional category (Table S2). Conversely, the large set of 341 down-regulated genes in response to heat included enriched functions for cell wall modification, synthesis and degradation, hormone metabolism (brassinosteroids and gibberelins), protein synthesis and various functions combined under "misc" (Fig. 4). Although "stress" associated

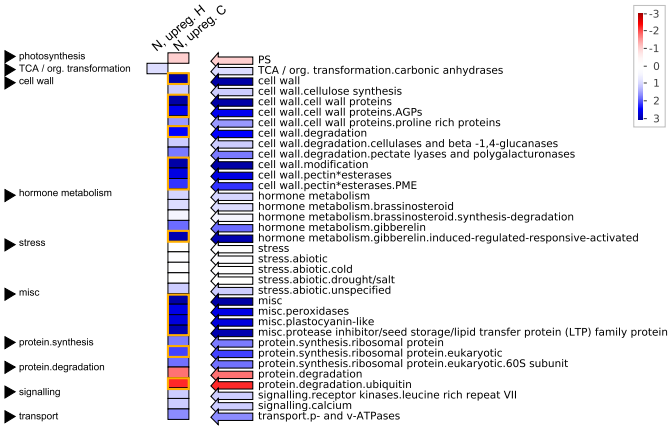

Fig. 4. Functional enrichment of differentially expressed genes in the northern *N. noltii* population. Functional categories are defined via MapMan (Usadel et al., 2009). Enrichment (blue)/depletion (red) of functional categories that are up-regulated in either treatment condition. N: northern population; H: heat treatment; C: control treatment. Coloring represents z-scores of FDR corrected p-values of the enrichment test. Boxes framed in orange exceed the significance threshold of FDR $\alpha < 0.05$.

functions were not significantly enriched, various subcategories were present [Fig. 4; "stress.abiotic": 1 gene (osmotin 34), "stress.abiotic.cold": 2 genes, "stress.abiotic.drought/salt": 4 genes, "stress.abiotic.heat": 1 gene (heat-shock protein binding) "stress.abiotic.unspecified": 4 genes] (Table S2).

3.5. Role of HSP expression in *Z. marina* and *N. noltii*

Shoots from both species displayed decreased shoot counts in response to heat stress (see Section 3.6 "Effects of the heat wave simulation on population performance"). We therefore investigated the role of HSP expression in both species, as HSPs are well known markers for heat stress. For each species, expression profiles for all 78 genes annotated with the functional term "stress.abiotic.heat" of all four libraries were compared with the constructed maximum and minimum expression profile of the respective species via MDS analysis. These constructed maximum (minimum) expression profiles of HSP genes for each species were obtained by taking the maximum (minimum) expression value of each gene out of the four respective libraries.

For *N. noltii*, none of the libraries grouped with the maximal expression profile (Fig. S6A). In contrast, heat-treated libraries of *Z. marina* showed a clear grouping with the constructed maximal expression profile, while control libraries were more similar to the minimum expression profile (Fig. S6B). This suggests that while HSPs were up-regulated under the simulated heat at 26 °C in *Z. marina*, no up-regulation of well-known members of the heat shock protein family occurred in *N. noltii*.

The within-species comparison for *N. noltii* revealed up-regulation of 28 genes in response to heat in the northern *N. noltii* population, none of them encoding HSPs or genes of any functional category associated with the term "stress". To investigate whether the 28 genes were also important during the heat response of the southern population, the normalized expression profiles were compared between all four *N. noltii*

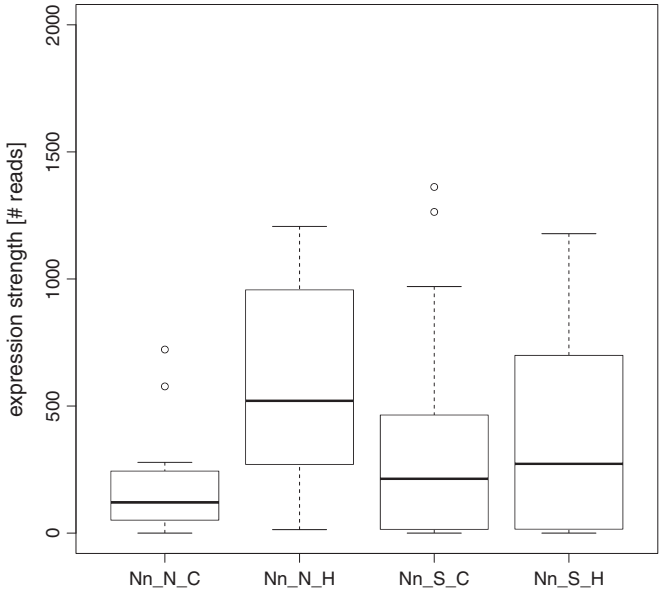

Fig. 5. Expression strength of the 28 genes that were up-regulated in response to heat in the northern population of *N. noltii*. Y-axis: normalized expression strength. X-axis: 4 cDNA libraries of *N. noltii* (Nn), populations: northern (N), southern (S); heat treatment (H), control treatment (C). Nn_N_C: exclusion of the 2 greatest values [max. 5451], Nn_N_H: exclusion of the 4 greatest values [max. 26,527], Nn_S_C: exclusion of the 3 greatest values [max. 7007] and Nn_S_H: exclusion of the 4 greatest values [max. 13,240] in the figure for displaying purpose. Expression of all 28 genes is different between libraries in the following order Nn_N_C < Nn_S_C < Nn_S_H < Nn_N_H (FDR $\alpha < 0.05$; paired Wilcoxon test, one sided for each of the 3 pairwise comparisons between libraries).

libraries. While the expression of the 28 "heat response" genes was in general strongest during heat in the northern population, they show intermediate expression levels in both southern *N. noltii* libraries (Fig. 5; FDR $\alpha < 0.05$, Fig. S7). This suggests an increased constitutive expression in the southern population for the 28 genes of the northern heat response.

3.6. Effects of the heat wave simulation on population performance

Population performance in response to the heat wave was measured using normalized changes in shoot abundance. A generalized linear model (GLM) approach showed significant treatment and time point effects for both species (p-value < 0.05) with a negative effect of the heat treatment and a greater shoot loss towards the end of the experiment (Table S3). For *Z. marina*, the negative effect of the heat treatment was weakest during acute heat on the northern population; the southern population performed better throughout the experiment (p-value < 0.05) (Fig. S8, Table S3). For *N. noltii*, no significant difference was found in performance between populations (p-value < 0.05, Table S3). The treatment effect was weakest during acute heat in the northern population (Fig. S8). Short-term reductions in growth were present in both species.

4. Discussion

In accordance with the expectation of *N. noltii* being more stress tolerant, we observed a higher temperature threshold for the induction of heat shock proteins in *N. noltii* compared to *Z. marina*, regardless of population origin. Moreover, we identified a higher constitutive expression of heat responsive (HR) genes in populations from the southern location of both species, suggesting a possible mechanism for local adaptation.

4.1. Heat response in Z. marina

Our study supports earlier work on *Z. marina* showing a largely concordant acute heat stress response between populations from northern and southern European locations and the expected up-regulation of several heat shock proteins upon heat treatment (Franssen et al., 2011a) (Table S4, Fig S6). Across locations, HSP up-regulation in *Z. marina* indicates molecular stress during the realistic heat wave scenario at water temperatures of 26 °C (see also Bergmann et al., 2010), which is further supported by detrimental effects on shoot abundance as well as reduction in growth rates and poorer photosynthetic performance shown in previous experiments (Bergmann et al., 2010; Winters et al., 2011; Gu et al., 2012).

Heat stress responses, however, involve many thermal tolerance processes other than induction of HSP genes (Krebs, 1999; Larkindale et al., 2005; Wahid et al., 2007; Kotak et al., 2007; Gu et al., 2012). Additional functional gene categories up-regulated during heat stress were identified in our novel approach using 56-fold more RNA-seq reads compared to earlier work (Franssen et al., 2011a) (for gene list see Table S2). Among the up-regulated genes were FKBPs (FK506-binding proteins), which are immunophillins involved in protein folding, signal transduction and chaperone activity (Aviezer-Hagai et al., 2007). FKBPs interact with HSP90 in *A. thaliana* (Rotamase FKBP1, see Table S2) (Aviezer-Hagai et al., 2007) or protect cells from oxidative stress (Gallo et al., 2011). Also up-regulated were several components of the 30S and 50S subunits of the chloroplast ribosomes, which are involved in the translation of chloroplast encoded genes (Nicolaï et al., 2007). However, no up-regulation of chloroplast genes involved in photosynthesis pathways, lipid acid synthesis, or translation/transcription machinery (Wicke et al., 2011) was detected.

In *Z. marina*, genes related to cell wall modifications were up-regulated, particularly pectin esterases and xyloglucan endotransglucosylases, (Table S2), the latter important for secondary cell wall reinforcement after the completion of cell expansion (Bourquin et al., 2002). Similar up-regulation of both classes of cell wall-related proteins has been observed in Chinese cabbage in response to mild heat treatment, leading to increased cell wall thickness and thermotolerance (Yang et al., 2006). In summary, heat expression responses in *Z. marina*, besides HSPs, included protectors against oxidative stress and genes that may increase thermotolerance via fortification of secondary cell walls.

4.2. Heat response in N. noltii

Expression profiles of *N. noltii* were more divergent among populations from the northern and southern location compared to *Z. marina*. While *N. noltii* from the southern location showed a weak expression response to the heat treatment, a large change in gene-expression was observed in the northern *N. noltii*, mainly due to the down-regulation of genes during heat treatment. In contrast to *Z. marina*, where genes involved in cell wall modification were up-regulated in response to heat, *N. noltii* showed a down-regulation of various genes involved in cell wall modification and degradation under heat treatment. While this seems contradictory, it might be explained by different optimal temperatures of both species. *Z. marina*, which typically occurs in colder waters, might require heat "protection" through cell wall fortification (Yang et al., 2006). In contrast, *N. noltii* commonly in warmer waters has adjusted to higher temperatures constitutively but experiences negative tradeoffs of this "heat protection" in colder waters, which in turn requires cell wall degradation and modification. Such a hypothesis, however, remains speculative and requires experimental validation.

Importantly, up-regulation of HSP genes was detected in neither *N. noltii* population (Table S2), although *N. noltii* (as did *Z. marina*) showed reduced shoot growth in response to heat. The results were surprising because HSP up-regulation typically occurs during stress (including heat stress) and accordingly, has been used as a bio-indicator for stress-conditions (Wahid et al., 2007; Kotak et al., 2007).

4.3. Differences in thermal thresholds between both species

Two hypotheses may explain the lack of HSP up-regulation in *N. noltii*. First, HSP expression may have been up-regulated earlier in the heat wave experiment and decreased while the stress-temperatures continued; or secondly, the critical temperature threshold was not reached. Evidence supporting the first hypothesis has been found in *N. noltii* (and *A. thaliana*) at 38 °C, where HSP expression returned to pre-stress levels within several hours or days after heat stress was initiated (but before it was removed) (Massa et al., 2011). Conversely, HSP up-regulation in *Z. marina* can persist for 1–3 weeks with a constant applied stress at only 26 °C (Bergmann et al., 2010; Franssen et al., 2011a). The mechanisms behind recovery to pre-stress HSP expression levels during stress exposure vs. ongoing induction are not well studied and it is not known to what extent this effect depends on the strength of the applied heat stress.

Regarding the second hypothesis, the lack of HSP induction for *N. noltii* is due to a higher temperature threshold for HSP up-regulation relative to *Z. marina*. This correlation between habitat temperature and HSP up-regulation might be an indicator for different ecological niches, a phenomenon commonly observed between species pairs (summarized in Feder and Hofmann, 1999). Numerous examples include fucoid seaweeds (Jueterbock et al., 2014), mussels (*Mytilus*), marine snails (*Tegula*), fruit flies (*Drosophila*), ants (*Cataglyphis* and *Formica*), yeast (*Saccharomyces*) (Feder and Hofmann, 1999), lizards (Ulmasov et al., 1992) and shrubs (*Prunus* and *Ceanothus*) (Knight, 2010), where congeners and/or related species occur in different ecological niches such as upper vs. lower intertidal areas (Feder and Hofmann, 1999), south vs. north facing slopes (Knight, 2010) or different climatic zones (Ulmasov et al., 1992; Gehring and Wehner, 1995; Hofmann and Somero, 1996; Krebs, 1999). In each case, the species

naturally occurring in the environment with higher temperatures have higher HSP induction thresholds, which usually differ by 2–7 °C (Ulmasov et al., 1992; Hofmann and Somero, 1996; Feder and Hofmann, 1999). For the *Z. marina* and *N. noltii* species pair, where long term heat treatment at 25 °C showed over-expression of HSPs in *Z. marina* (also see Bergmann et al., 2010; Franssen et al., 2011a), but not in *N. noltii*, the only additional study on *N. noltii* showed HSP up-regulation in response to a simulated low tide at ~38 °C (Massa et al., 2011). Thus, the exact difference in HSP induction thresholds in *Z. marina* and *N. noltii* remains unknown.

The lack of HSP induction in *N. noltii* at 26 °C, in contrast to *Z. marina*, may be adaptive. Several studies have shown that over-expression of HSP genes can be costly in terms of fertility, growth, development and survival and are only up-regulated when benefits outweigh costs (Bettencourt et al., 1999; Sørensen et al., 2003; Sørensen, 2010). HSP expression is known to be induced by denatured proteins (Ananthan et al., 1986; Krebs, 1999). Thus, the lack of HSP up-regulation in *N. noltii* suggests that 25 °C were too low to induce protein denaturation. A higher temperature threshold for protein denaturation can be achieved through protein stability by 1) intrinsic factors such as amino-acid composition and 2) extrinsic factors besides HSPs such as thermostabilizing solutes (Fields, 2001), e.g. 2,3-diphosphoglycerate in methanogenic bacteria (Hensel and König, 1988) or sugars as protective osmolytes in seagrasses (Gu et al., 2012). While thermostabilizing solutes enable more plastic responses by increase or decrease of the respective solutes, intrinsic protein properties require a multitude of microevolutionary changes, e.g. changes in amino-acid composition, which only arise over much greater time scales (Fields, 2001). As both species co-occur in a wide range of habitats, extrinsic factors seem more likely to influence protein stability in both species; however, this requires further experimental investigation.

4.4. Microevolutionary differences between populations from different temperature regimes

The seagrass populations from northern and southern European locations were chosen not only to provide biological replication to infer species differences, but also to gain insights into population differences from colder (northern) vs. warmer (southern) temperature habitats (Fig. S1). A common-stress-garden setup with a relatively long acclimation phase (~50 days) was chosen to minimize non-heritable components induced by the native habitat (Hoffmann et al., 2005; Whitehead and Crawford, 2006). Population responses to heat were similar for *Z. marina* from both locations with 267 genes concordantly up-regulated during heat and very divergent in *N. noltii* with 28 genes up-regulated in the northern strongly responding population. The respective heat responsive (HR) genes showed signs for a constitutive up-regulation in the southern population of both species. This suggests that constitutive up-regulation of HR genes in a species might be an adaptive mechanism of populations from different local temperature regimes to cope with elevated habitat temperatures, which can in general occur over microevolutionary time scales (Bettencourt et al., 1999).

A similar pattern with a higher constitutive expression of HSPs in species from habitats with higher characteristic temperatures was observed among species of lizards (Ulmasov et al., 1992; Zatsepina et al., 2000) and ants (Gehring and Wehner, 1995), although such a pattern may not be general (e.g. see Bettencourt et al., 1999; Zatsepina et al., 2000; Barua et al., 2008). Besides the constitutive up-regulation of HR genes, the strength of the inducible response might also play an important role (e.g. Bettencourt et al., 1999; Feder and Hofmann, 1999). In *Z. marina*, the inducible heat response was stronger in the population from the southern location and even exceeded the observed difference in the constitutive expression change. However, such a pattern was not observed for *N. noltii*. While these inter-species differences still require further study to verify or falsify their adaptive nature, our results illustrate the importance of inter-population variability of response, i.e., variation in the amplitude and duration of transcriptional responses.

4.5. RNA-seq analysis in non-model organisms

Our inter-species transcription analysis relied on RNA-seq with subsequent mapping to a de novo assembly of a reference transcriptome, the quality of which has a significant impact on the accuracy and resolution of the subsequent expression analysis (Martin and Wang, 2011). Although a growing number of de novo transcriptome assemblies, based on RNA-seq data, have been performed for higher plants (e.g. Vega-Arreguin et al., 2009; Wang et al., 2009; Franssen et al., 2011a,b) and improvements in assembly software have been made, de novo assembly of higher eukaryotes remains a challenging task (Martin and Wang, 2011). Whenever a reference genome is available, remapping approaches are used to guide the transcriptome assembly (Guttman et al., 2010; Robertson et al., 2010; Trapnell et al., 2010; Martin and Wang, 2011). Because of the current state of the art and the features of redundancy observed in the de novo assemblies of *Z. marina*, *N. noltii*, and previous studies (Martin et al., 2010; Franssen et al., 2011b; Grabherr et al., 2011; Martin and Wang, 2011; Mundry et al., 2012), gene identification via orthology to the well annotated reference species *A. thaliana* was chosen.

4.6. Ecological transcriptomics

Our study provides a number of transcriptomic insights into the concept of functional ecological types. We suggest that the absence of an HSP up-regulation during the heat wave simulation is a molecular indicator for the ecological niche of *N. noltii*, which dominates intertidal habitats, in which extreme temperatures of 36 °C may be experienced during tidal exposure (Massa et al., 2008). *Z. marina*, in contrast, dominates in more thermally stable subtidal habitats with fewer extreme temperatures and temperature variances. Therefore, extreme temperatures do not explain the dominance of *Z. marina* in subtidal areas, whereas they may explain the absence of *Z. marina* in the intertidal. Possible causative factors may include competition for light or a competitive advantage of the taller *Z. marina* in more stable subtidal environments (Borum et al., 2004). The latter factor is also in accordance with the C-S-R triangular diagram of Grime (Grime, 1977), which groups the characteristics of species in relation to competitive ability, stress tolerance and dispersal capability (weediness). Under this categorization intertidal *N. noltii* has been classified as a stress-tolerant ruderal, while subtidal *Z. marina* populations are classified as competitors (Phillips et al., 1983; Phillips and Menez, 1988).

In general the effects of global climate change, including increased temperatures and more frequent and/or stronger occurrences of extreme weather events will result in range shifts, local extinction or adaptation (Easterling et al., 2000; Lohbeck et al., 2012). The molecular signals during the simulation of the heat wave scenario suggested that extreme temperature events (Easterling et al., 2000) will interfere with current species interaction hierarchies. For example, existing competitive advantages of *Z. marina* over *N. noltii* may decrease, which could impact other community interactions and result in new community assemblies. With growing "omics" resources to explore the roles of transcriptional diversity, our understanding of molecular and functional diversity will help to redefine our understanding of ecological concepts (Procaccini et al., 2012; Mazzuca et al., 2013).

Author contributions

J.L.O., T.B.H.R., and E.B.B. designed the research; S.U.F., J.G., G.W., A.K.H., I.W., M.S. and J.A.C. performed the experimental research; S.U.F., J.G., T.B.H.R., and E.B.B. analyzed the data; and S.U.F., E.B.B., J.L.O., J.A.C., and T.B.H.R. interpreted the data and wrote the paper.

Data accessibility

Raw reads of 454 and Illumina sequencing are accessible at NCBI SRA (accession number of the complete study: SRP022957 including two 454 and eight Illumina libraries). The de novo assembly of the *N. noltii* transcriptome is available at: http://drzompo.uni-muenster.de/downloads, library: Nano_A.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.margen.2014.03.004.

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