Thermal stress resistance of the brown alga *Fucus serratus* along the North-Atlantic coast: acclimatization potential to climate change

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Abstract

Seaweed-dominated communities are predicted to disappear south of 45°latitude on North-Atlantic rocky shores by 2200 because of climate change. The extent of predicted habitat loss, however, could be mitigated if the seaweeds' physiology is sufficiently plastic to rapidly acclimatize to the warmer temperatures. The main objectives of this study were to identify whether the thermal tolerance of the canopy-forming seaweed *Fucus serratus* is population-specific and where temperatures are likely to exceed its tolerance limits in the next 200 years. We measured the stress response of seaweed samples from four populations (Norway, Denmark, Brittany and Spain) to common-garden heat stress (20 °C –36 °C) in both photosynthetic performance and transcriptomic upregulation of heat shock protein genes. The two stress indicators did not correlate and likely measured different cellular components of the stress response, but both indicators revealed population-specific differences, suggesting ecotypic differentiation. Our results confirmed that thermal extremes will regularly reach physiologically stressful levels in Brittany (France) and further south by the end of the 22nd century. Although heat stress resilience in photosynthetic performance was higher at the species' southern distributional edge in Spain, the *hsp* expression pattern suggested that this edge-population experienced reduced fitness and limited responsiveness to further stressors. Thus, *F. serratus* may be unable to mitigate its predicted northward shift and may be at high risk to lose its center of genetic diversity and adaptability in Brittany (France). As it

is an important intertidal key species, the disappearance of this seaweed will likely trigger major ecological changes in the entire associated ecosystem.
Keywords: global warming, heat stress, macroalgae, heat shock protein, photosynthetic performance

1. Highlights

- *F. serratus* shows patterns of local thermal adaptation
- *F. serratus* will experience stressful temperatures in Brittany and Spain by 2200
- *F. serratus* may become extinct from the North-Iberian Peninsula under climate change

2. Introduction

2.1. Increasing thermal stress in the North-Atlantic intertidal

Heat waves have become more frequent and extreme throughout the 20th century and are predicted to increase in the 21st century (Easterling et al., 2000; Meehl et al., 2007). On a global scale, species are responding to thermal stress with phenological changes and distributional range shifts that often involve local extinction (Hickling et al., 2006; Walther et al., 2002). The response of marine rocky intertidal species is often considered an early warning signal of climate change (Pearson et al., 2009) since they generally live close to their upper thermal tolerance limits and have low potential to respond to further rising temperatures (Somero, 2010; Tomanek, 2010). Intertidal species along North-Atlantic shores will experience up to 4 °C warmer water temperatures by the end of the 21st century (Müller et al., 2009) and a 5 to 10 times higher frequency of heat waves within the next 40 years (Barriopedro et al., 2011; Schär et al., 2004). In order to better understand the impact of increasing numbers of heat waves upon rocky intertidal shores, it is important to investigate the acclimatization potential of foundational key species (sensu Dayton, 1972) that play a pivotal role for the structure of the intertidal rocky-shore community.

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2.2. An intertidal key species under thermal stress

The brown seaweed *Fucus serratus* provides habitat and food for a highly diverse community of species (Fredriksen et al., 2005), thus playing a key role in the Northeast-Atlantic rocky intertidal where it inhabits rocky shores from northern Portugal to northern Norway (Lüning et al., 1990). On the Northwest-Atlantic coast, *F. serratus* was introduced to Nova Scotia (Canada) 100-150 yrs ago (Brawley et al., 2009). A recent study predicted that *F. serratus*, together with two other macroalgal key species (*F. vesiculosus* and *Ascophyllum nodosum*), will disappear by 2200 from North-Atlantic shores south of 45 ° latitude under projected climate change (Jueterbock et al., 2013).

While the North-Iberian Peninsula is one of three putative glacial refugia where *F. serratus* survived the Last Glacial Maximum (18-20 kya) (Hoarau et al., 2007), its within-population genetic diversity eroded during thermally induced cycles of range contractions and expansions (Coyer et al., 2003). This may impede phenotypic plasticity and adaptive evolvability (Bijlsma and Loeschcke, 2012) and thus could explain maladaptation to warm thermal stress in northern Portugal (Pearson et al., 2009) and inhibition of growth, physiological performance (Martínez et al., 2012) and reproductive capacity (Arrontes, 1993; Viejo et al., 2011) by extreme summer temperatures in northern Spain.

In contrast, the other two refugia, Southwest-Ireland and Brittany, are hot-spots of genetic diversity (Coyer et al., 2003; Hoarau et al., 2007) and thus may be more resilient to climate change (Ehlers et al., 2008). Moreover, the low dispersal potential and small-scale genetic differentiation of *F. serratus* (Coyer et al., 2003) might favor local thermal adaptation (Hampe and Petit, 2005). Thermal acclimatization and local thermal adaptation are crucial factors to assess a species' extinction risk under climate change but their geographical pattern along the distributional range of *F. serratus* are presently unknown.

2.3. Physiological acclimatization to thermal extremes

A universal strategy of molecular acclimatization to stressful temperatures is the heat shock response (HSR), which involves the transcriptional up-regulation of heat shock proteins (HSPs). HSPs act as molecular chaperones and protect the organism from inappropri-

ate interactions of denatured or aggregated non-native proteins (Feder and Hofmann, 1999). Some HSP forms can be used as universal stress biomarkers since their genes are highly conserved among widely disparate species and their expression level is induced by different forms of environmental stress (Feder and Hofmann, 1999). The response is, however, limited by the corresponding energetic costs and cytotoxic effects it involves (reviewed in Feder and Hofmann, 1999; Sørensen and Loeschcke, 2007).

Photosynthetic performance is another sensitive indicator of thermo tolerance in photosynthetic organisms, as photosynthesis is specifically sensitive to heat stress (Berry and Bjorkman, 1980). Photosystem II (PS II) was shown to be affected first, with warm temperatures negatively incluencing carbon metabolism and electron transport in the photosynthetic apparatus (Berry and Bjorkman, 1980).

2.4. Objectives

The main aim of this study was to identify whether the acclimation potential of *F. serratus* could mitigate its predicted extinction from shores south of 45 °N under climate change scenarios. More specifically, we addressed three questions:

1. Is photosynthetic performance and *hsp* expression of *F. serratus* under acute heat stress population-specific, thus indicating local adaptation?
2. How is individual variation in *hsp* gene expression correlated with photosynthetic performance?
3. Where will temperatures rise over the next 200 years beyond the thermal tolerance limits of *F. serratus* and thus threaten it with extinction?

3. Materials and methods

3.1. Common garden heat stress experiments

We collected $\geqslant$30 adult individuals of *F. serratus* from four locations covering the species' latitudinal range of distribution (see Figure 1 and mapped sampling sites in supplementary material S8) during a span of four weeks in May/June 2011: 1) Kirkenes, Norway (69° 47'

24.36" N, 30° 47' 26.94" E), 2) Blushøj, Denmark (56° 10' 1.56" N, 10° 43' 57.98" E), 3) Roscoff, Brittany (48° 42' 46.71" N, 4° 1' 18.62" W), and 4) La Coruña, Spain (43° 21' 59.14" N, 8° 23' 17.51" W). The individuals were transported to the wetlab facilities of the University of Nordland in Mørkvedbukta (Bodø, Norway) and placed in one of two aquaria (1m x 1m x 0.5m, Norwegian and Danish samples in one, Brittany and Spanish samples in the other) within 1 - 2 days after collection, then acclimated for >4 weeks to ca. 9 °C running natural seawater (both aquaria connected with the same water flow-through), a 16:8 h L:D cycle, and 40 - 70 µmol photons m^{-2} s^{-1} (OSRAM Fluora, 150 Watt). Common-garden heat stress experiments were conducted from July to December 2012, consisting of 4 apical tips (ca. 5cm) cut from each of 6 - 10 individuals in each population. Three of the 4 tips were transferred for 1h to aquaria in which water temperature was increased.

We applied 5 stress temperatures in 5 independent experiments with longer acclimation times for the experiments that were carried out later in the year: 1) 20 °C stress after 8 weeks of acclimation, 2) 24 °C stress after 7 weeks of acclimation, 3) 28 °C stress after 23 weeks of acclimation, 4) 32 °C stress after 7 weeks of acclimation, and 5) 36 °C stress after 8 weeks of acclimation. Temperatures ≥24 °C exceed the maximum in situ water temperatures experienced by *F. serratus*, even at its southern distribution limit (Martínez et al., 2012; Pearson et al., 2009), but *Fucus* canopy-temperatures can exceed 30 °C during summer in North-Portugal (Pearson et al., 2009). With the selected stress temperature range (20 °C - 36 °C), we aimed for a forced response covering the stressful to thermal temperature limits of all four populations in order to identify population-specific differences in photosynthetic performance and gene expression. One tip per individual was used to measure photosynthetic performance and heat shock protein gene expression from the same 6 - 10 individuals at 4 different time points: 1) before heat stress (control, 1^{st} tip), 2) after 15 min heat stress (2^{nd} tip), 3) after 60 min of heat stress 3^{rd} tip), and 4) after 24 h recovery at 9 °C (4^{th} tip).

3.2. Photosynthetic performance

We measured from each sample (3 measurements/sample) the increase in chlorophyll a fluorescence upon illumination after a ≥15 min dark period (OJIP curve (Bussotti et al.,

2010), also called the Kautsky effect (Kautsky (1960) in Maxwell and Johnson, 2000)) with a PAM-Fluorometer (FluorPen FP100, Photon Systems Instruments) using a saturating pulse of 73%. From these measurements, we extracted the performance index (Pi_{ABS}) (Strasser et al., 2000) reflecting the functionality of PS II and photosynthetic performance in general (Bussotti et al., 2010; Stefanov et al., 2011; Živčák et al., 2008) by combining three parameters: 1) the density of reaction centers, 2) the electron transport at the onset of illumination, and 3) the maximum energy flux reaching the reaction center in PS II. Pi_{ABS} is calculated as follows: $Pi_{ABS} = \frac{1-(F_0/F_M)}{M_0/V_J} x \frac{F_M-F_0}{F_o} x \frac{1-V_J}{V_J}$, where F_0 is the minimal fluorescence intensity in a dark adapted frond when all reaction centers are opened (all quinone acceptors are oxidized and can accept electrons), F_J is the fluorescence intensity at 2 ms illumination, F_M is the maximum fluorescence intensity when all reaction centers are closed (all quinone acceptors are reduced), V_J is relative variable fluorescence at 2 ms calculated as $V_J = (F_J F_0)/(F_M F_0)$, and M_0 reflects the initial slope of fluorescence kinetics, calculated as $M_0 = 4 * (F_{300\mu s} F_0)/(F_M F_0)$ (Živčák et al., 2008).

To test for potential maternal or genetic effects on photosynthetic performance, we compared Pi_{ABS} values between the control samples (acclimated to 9 °C for $\geq$4 weeks) of each of the four populations. We calculated estimators of nonparametric Tukey contrast effects and associated p-values using the function "nparcomp" with the R package 'nparcomp' (Konietschke, 2012).

We normalized the Pi_{ABS} (arithmetic mean of 3 measurements taken from each sample) by dividing the mean Pi_{ABS} values of each sample through the mean Pi_{ABS} values measured from the control sample of the same individual. Values >1.5 times the inter-quartile range in box plots for each combination of stress temperature, population, and time point, were removed from the dataset if the Grubbs' test (R package 'outlier' (Komsta, 2011)) identified them as significant outliers (see S1 in the supplementary material for outlier values that were not considered in the data analysis).

We tested for significant differences in normalized photosynthetic performance between populations and time points using a nonparametric analysis of repeated-measures (the same individuals were measured over time) with the "f1.ld.f1" function of the software package

'nparLD' (Noguchi et al., 2012) in the statistical program R 3.0.2 (R Development Core Team, 2013). In case of significant time point effects (see Table S2 in the supplementary material), we tested if the average normalized Pi_{Abs} values at the three time points (15 min heat stress, 60 min heat stress and 24 h recovery) were significantly different from the controls by calculating for each population 95% bootstrap confidence intervals in R 3.0.2 (R Development Core Team, 2013). We regarded the normalized performances significantly different from the controls if they did not include the value 0. In case of a significant population or interaction effect (see Table S2 in the supplementary material), we calculated Tukey contrast effects of normalized Pi_{Abs} values between the four populations (Norway, Denmark, Brittany, and Spain) for each time point (15 min heat stress, 60 min heat stress and 24 h recovery) using the function "nparcomp" with the R package 'nparcomp' (Konietschke, 2012).

3.3. Heat shock protein gene expression

3.3.1. RNA extraction and cDNA synthesis

Controls and stressed *Fucus* samples were placed in liquid nitrogen immediately after fluorescence measurements and stored at -80 °C before lyophilization for a maximum of 3 weeks. RNA was extracted from the lyophilized samples of the 28 °C and 32 °C heat stress experiments (at which we found population-specific differences in photosynthetic performance) as described in Pearson et al. (2006). Samples were purified with the ZR-96 RNA Clean & Concentrator kit (Zymo Research, Irvine, USA) and potential PCR inhibitors were removed with the OneStep-96TM PCR Inhibitor Removal Kit (Zymo Research). RNA concentrations were quantified with the Qubit RNA Assay kit (Life Technologies, Paisley, UK) using a Qubit 2.0 Fluorometer (Life Technologies) and RNA integrity was verified by agarose gel electrophoresis. The extracted RNA was of sufficient quantity and quality for 8 individuals/population (28 °C stress) and 4–6 individuals/population (32 °C stress), respectively. Extracted RNA was reverse-transcribed to cDNA in 20 µl reactions with the QuantiTect Reverse Transcription Kit (Quiagen, Hilden, Germany) using a Veriti 96-Well Fast Thermal Cycler (Life Technologies). All 32 °C stress samples and the Danish 28 °C stress samples

were reverse transcribed together with a starting amount of RNA of 66.0 ng, while the Spanish, Brittany and Norwegian 28 °C stress samples were reverse transcribed with a starting amount of RNA of 40.0 (5 samples with 22.6 ng due to their specifically low concentration). We corrected for these quantitative differences in the data analysis (described below).

3.3.2. Real-time PCR

The qPCR reactions were performed in a StepOnePlus real-time PCR System (Life Technologies) using primers (Table 1) designed with the Primer Express 3.0 software (http://primer-express.software.informer.com/3.0/). The primers (Table 1) were designed from EST libraries of heat stressed *Fucus* (Pearson et al., 2010) and targeted unique *hsp* genes based on the ESTs. However, as more than 10 *shsp*, three *hsp90*, and two *hsp70* genes were identified in *Fucus*, we can not fully exclude the possibility that we have amplified more than one member of the same gene family. The total reaction volume was 5 µl, containing 2.5 µl Fast SYBR Green Master Mix (Life Technologies, Paisley, UK), 2 µl cDNA (1:20 dilution) and 0.5 µl of a solution containing forward and reverse primers at 5 µM each. All samples were run in duplicate and equimolar pools of cDNA served as positive controls and minus reverse transcriptase (-RT) controls, while no template controls were run to test for contamination. The PCR amplification protocol consisted of 95 °C for 20 sec followed by 40 cycles of 95 °C for 3 sec and 62 °C for 30 sec (for all primers). To verify the amplification specificity, we performed a melting curve analysis from 60 °C to 95 °C. The cDNA was successfully quantified in 4–6 individuals per population and gene for the 32 °C stress samples and for 6–8 individuals per population and gene for the 28 °C stress samples.

Dilution series (1:5 dilution/step; from 1:1 to 1:625) of the cDNA pools (1:20 dilution) were amplified in duplicate and served to calculate the PCR amplification efficiency E from the regression slope of the threshold cycle (Ct) versus log10 cDNA concentration after Pfaffl et al. (2002). To normalize the expression quantities, based on the expression level recorded for the two housekeeping genes from the same sample, we used the R package 'SLqPCR' (Kohl, 2007) that implements the normalization method described in Vandesompele et al. (2002).

3.3.3. Statistical analysis

To test for potential maternal or genetic effects on gene expression levels, we compared relative normalized expression quantities between the control samples (acclimated to 9 °C for ≥4 weeks) of each of the four populations using ANOVA on log-transformed values (due to non-normality based on the Shapiro-Wilk normality test), followed by Tukey's post-hoc tests in R 3.0.2 (R Development Core Team, 2013). To test whether the acclimation period had an effect on *hsp* expression patterns, we included "acclimation period" as an additional explanatory variable that discriminated the 28 °C stress control samples (control group 1, acclimated for 23 weeks to 9 °C) from the 32 °C stress control samples (control group 2, acclimated for 7 weeks to 9 °C). We calculated the fold-change of gene expression by dividing the relative normalized expression quantities of each sample through the control sample values of the same individual. Potential outliers were removed if log-transformed or fold-change values were >1.5 times the inter-quartile range above the 3rd quartile or below the 1st quartile (see S3 and S4 in the supplementary material for outlier values that were not considered in the data analysis).

For each heat shock protein gene (*hsp70*, *hsp90*, and *shsp*) we tested for significant differences in fold-change expression between populations and time points using a nonparametric analysis of repeated-measures (the same individuals were measured over time) with the "f1.ld.f1" function of the R package 'nparLD' (Noguchi et al., 2012). In case of a significant population or interaction effect (see S5 in the supplementary material), we calculated Tukey contrast effects of fold change expressions between the four populations (Norway, Denmark, Brittany, and Spain) for each time point (15 min heat stress, 60 min heat stress and 24 h recovery) using the function "nparcomp" with the R package 'nparcomp' (Konietschke, 2012).

3.4. Relation between photosynthetic performance and hsp gene expression

We tested for correlations between the individual change in the photosynthetic performance (relative normalized Pi_{Abs} values) and relative normalized *hsp* gene expression quantities (first explanatory variable) after 15 min and 60 min heat stress (28 °C and 32 °C)

with ANCOVAs using linear models in R 3.0.2 (R Development Core Team, 2013). Models were performed separately for the three *hsp* genes (*hsp70*, *hsp90* and *shsp*) and the factor "population" was included as second explanatory variable to test for population-specific effects.

3.5. Thermal regime

To characterize the thermal regime at the four sampling sites (Fig. 1) under present-day conditions and over the next two centuries, we extracted annual means, minima and maxima of monthly averaged sea surface temperature (SST) and of monthly averaged surface air temperature (SAT) from GIS rasters of the Bio-ORACLE database (http:// www.oracle.ugent.be/ index.html) using the R package 'raster' (Hijmans and van Etten, 2011). Rasters of present-day SST grids are described in Tyberghein et al. (2012), rasters of present-day SAT grids and predicted SAT and SST (based on the A1B IPCC climate change scenario (720ppm stabilization) and the UKMO-HadCM3 model (Gordon et al., 2000; Johns et al., 2003)) are described in Jueterbock et al. (2013)). Although body temperatures of intertidal organisms can differ broadly from low-tide air temperatures (Helmuth, 2009; Helmuth et al., 2006), we believe that our estimations of average SST and SAT of the warmest month provided rough proxies for the frequency of warm temperature extremes (higher averages = higher frequency).

4. Results

4.1. Photosynthetic performance

Photosynthetic performance did not differ significantly between the populations under control conditions (9 °C, see S6 in the supplementary material). Photosynthetic performance decreased significantly ($p \leq 0.05$) at stress temperatures ≥24 °C (significant "Duration" effect in S2) in all four populations after an exposure time of 60 min (Figure 2e). After an exposure time of 15 min, the Norwegian population showed a significant Pi_{ABS} decrease only at ≥28 °C (Figure 2g), while the performance of all other populations decreased significantly at ≥24 °C (Figure 2d).

Only the Spanish population recovered from 32 °C stress after 24 h recovery at 9 °C, indicated by an average Pi_{ABS} value that was not significantly different from control sample levels (Figure 2l). In contrast, the Norwegian, Danish and Brittany populations did not recover from >28 °C stress and the performance of the Brittany population remained significantly low after recovery from 24 °C stress exposure (Figure 2f).

Population-specific differences occurred at 20 °C, 24 °C and 36 °C, and interactions between population and duration (time point) were significant at 24 °C, 32 °C, and 36 °C (see S2). The Brittany population showed a significantly lower performance compared to the Spanish population after 24 h at 20 °C (Figure 2c). The Spanish population had a significant lower photosynthetic performance after 60 min at 24 °C compared to all other populations (Figure 2e) and compared to the Norwegian population after 15 min and 60 min at and 24 h recovery from 36 °C stress (Figure 2m,n,o).

4.2. Heat shock protein expression

The interaction between population and acclimation time was significant for the expression levels of all three *hsp* genes (see S7 in the supplementary material). The Danish population had significantly lower expression levels than any other population for *hsp70* and *hsp90* and lower *shsp* expression levels than the Brittany and Spanish samples for control group 1 (23 weeks of acclimation to 9 °C, Figure 3a,b)), but the pattern was not mirrored in control group 2 (7 weeks of acclimation to 9 °C, Figure 3d,e). The Spanish population showed significantly higher *hsp90* expression levels compared with the Norwegian and Danish populations in control group 1 (Figure 3b) and with the Norwegian and Brittany populations in control group 2 (Figure 3e). Furthermore, the Spanish population showed significantly higher *shsp* expression levels compared with all three other populations in control group 1 (Figure 3c) and with the Norwegian and Brittany populations in control group 2 (Figure 3f). The relative expression quantities differed between control group 1 and 2 for *hsp70* in the Norwegian and Brittany populations (Figure 3a,d) and for *hsp90* in all four populations (Figure 3b,e).

All three *hsp* genes showed a significant upregulation under 28 °C, but only the *hsp90*

and *shsp* genes responded significantly to 32 °C (no significant "Duration" effect for *hsp70* at 32 °C stress, see S5 in the supplementary material). No population-specific differences in the upregulation of *hsp90* gene expression were apparent (S5, and Figure 4g-l). A significant interaction between population and duration (time point) was found for the *hsp70* gene at 28 °C (see S5). The expression level of *hsp70* was significantly lower in the Spanish population than in the Norwegian or Danish populations after 60 min at 28 °C (Figure 4b).

Maximum transcriptional up-regulation (fold change in gene expression) was considerably higher for the *shsp* gene (max. 1000-fold change, Figure 4m-r) than for the *hsp70* gene (max. 2-fold change, Figure 4a-f) and the *hsp90* gene (max. 4-fold change, Figure 4g-l). Significant differences between populations were found for the *shsp* gene at 28 °C stress, but not at 32 °C stress (see S5 in the supplementary material). In the 28 °C experiment, the fold-change in *shsp* expression was significantly lower in the Spanish individuals than in the other three populations (Norway, Denmark, Brittany) (Figure 4o). In contrast, the Danish population responded to 28 °C stress with significantly higher fold-change in *shsp* expression than samples from Norway and Spain after 15 min (Figure 4m) and with higher fold-change than samples from any other population after 60 min and 24 h recovery (Figure 4n,o).

4.3. Relation between photosynthetic performance and hsp gene expression

The change in photosynthetic performance (ΔPi_{ABS}) was not significantly ($p \geq 0.05$) correlated with relative normalized expression quantities for any of the three *hsp* genes. The regression line slopes were insignificant at both 28 °C (15 min and 60 min: *hsp70* $p = 0.23$ and $p = 0.98$, *hsp90* $p = 0.94$ and $p = 0.58$, *shsp* $p = 0.84$ and $p = 0.75$), and 32 °C (15 min and 60 min: *hsp70* $p = 0.54$ and $p = 0.82$, *hsp90* $p = 0.92$ and $p = 0.66$, *shsp* $p = 0.16$ and $p = 0.98$).

4.4. Thermal regime

Under present-day conditions, the Danish and Spanish populations experience highest maximum SST and SAT (Figure 5a). In contrast, within the next two centuries, SST and SAT are predicted to reach highest maxima at the seaweed's southern range of distribution

in Brittany and Spain within the next 200 yrs. For the Brittany and Spanish populations, the average SST of the warmest month is predicted to rise nearly up to 24 °C, the minimum temperature with a significant negative fitness effect (Figure 2e).

5. Discussion

5.1. Hsp gene expression and loss of photosynthetic performance are not correlated

Increased expression levels of our three focal *hsp* genes did not mitigate the loss of photosynthetic performance under heat stress, as the two stress indicators varied independently from each other. One possible explanation for this lack of correlation is that the measured *hsp* gene transcription levels themselves do not necessarily correlate with translation and the presence of active, functional HSP proteins. Alternatively, the photosynthetic apparatus might be protected by other HSPs located in the stroma of the chloroplasts (cp-HSPs) (e.g. Downs et al., 1998). For example, cp-sHSP directly protect the electron transport and oxygen evolution of photosystem II (PS II)(Preczewski et al., 2000; Shakeel et al., 2012) and its upregulation was significantly positively correlated with photosynthetic thermotolerance of tomato (*Lycopersicon*) (Preczewski et al., 2000). This sHSP chaperone is also present in the chloroplast of symbiotic dinoflagellates of the genus *Symbiodinium* (Downs et al., 2000), but the role it plays in thermotolerance of brown seaweeds is poorly studied. In addition, other cellular components than HSPs can be involved in warm temperature acclimation (Collén et al., 2007). For example, detoxifying enzymes may protect PS II from damage by reactive oxygen species (ROS) and alteration of cell membrane lipid composition can secure functioning of photosynthesis under heat stress (Rowland et al., 2010). Thus, the three HSPs examined in the present study are unlikely to play a major role in protecting the photosynthetic apparatus of *F. serratus*. It appears that cellular *hsp* expression and photosynthetic performance measure different cellular processes in *F. serratus* and can not replace each other as heat stress indicators.

5.2. Population-specific heat-stress responses

5.2.1. Increased heat stress resilience in Spain

The Spanish population was more resilient to heat stress than the Norwegian, Danish and Brittany populations (recovery from up to 32 °C stress, Figure 2l). Its HSR revealed high constitutive gene expression (in *shsp* and partly *hsp90*, Figure 3b,c,e,f) but low inducible *hsp* gene expression (in some cases for *hsp70* (Figure 4b) and mostly for *shsp* Figure 4o,r). In combination, these *hsp* expression patterns indicate significant intrinsic differences (genetically or through maternal effects) between the Spanish and the other populations and suggest two alternative explanations for the population's increased heat stress resilience: local thermal adaptation or chronic thermal stress.

Local adaptation of *F. serratus* to warm temperatures is favored by its low dispersal potential and small-scale genetic differentiation (panmictic unit of ca. 2km) (Coyer et al., 2003) and thus may account for its increased heat stress resilience in Spain. Ecotypic differentiation in *HSP70* expression was for example found in *Drosophila melanogaster* that occurs in thermally selected *hsp70* variants (Bettencourt et al., 2002) and in phosphoglucose isomerase (PGI) genotypes of the leaf beetle *Chrysomela aeneicollis* (Dahlhoff et al., 2008). Increased thermostability of other than HSP proteins could lower the required *hsp* expression under heat stress (e.g. Barua et al., 2008), but this would not explain the high constitutive *hsp90* and *shsp* expression levels of the Spanish population under control conditions (Figure 3b,c,e,f). Thus, an adaptive shift in HSP chaperone performance to warmer temperatures is more likely to explain the reduced upregulation of *hsp* expression in the Spanish population under heat stress.

Heat-hardening under chronic high thermal stress levels is an alternative explanation for the constitutively high *hsp* expression of the Spanish population. Constitutively high expression of ATP-dependent *hsp* genes (in our case *hsp90*, since *shsp* is ATP-independent) involves metabolic costs at the expense of growth and reproduction (Feder and Hofmann, 1999; Sørensen and Loeschcke, 2007). Evidence that environmental stress can reduce growth comes from a study on the intertidal mussel *Mytilus californianus* demonstrating slower growth in the thermally stressful high intertidal (compared to the less stressful low inter-

tidal) (Hofmann, 2005) and from a study on the estuarine fish *Gillichthys mirabilis* where genes involved in protein synthesis, cell growth and proliferation were repressed in response to hypoxia (Gracey et al., 2001). Furthermore, repeated heat stress exposure reduced the fecundity of *Drosophila melanogaster* (Krebs and Loeschcke, 1994). Accordingly, reduced growth, reproductive capacity and physiological performance of Spanish southern edge populations of *F. serratus* (Martínez et al., 2012; Viejo et al., 2011) might be explained by a constitutive heat-stress response under chronic thermal stress.

Other than reducing fitness, warm-temperature acclimatization can inhibit responsiveness to further stress, as was found for heart function in porcelain crabs (genus *Petrolisthes*) (Stillman, 2003) and for general stress resilience in the Australian kelp *Ecklonia radiata* (Wernberg et al., 2010). The same inverse relationship between high *hsp* stock-levels (Figure 3b,c,e,f) and low inducible thermotolerance (lower *hsp70* and *shsp* up-regulation, Figure 4b,o,r) in our Spanish population was likewise found for the *hsp70* gene in the sea urchin *Strongylocentrotus purpuratus* (Osovitz and Hofmann, 2005) and is supported by the so-called "cellular-thermostat" model (reviewed in Tomanek, 2010). According to this model, stress conditions normally initiate the transcription of inducible *hsps* by the heat shock transcription factor 1 (HSF1), when the HSPs (e.g. HSP70 and HSP90) that hold HSF1 in an inactive state are required for protein stabilization and repair, but constitutively high HSP levels block this response since HSF1 is no longer released (Tomanek, 2010; Tomanek and Somero, 2002). Moreover, significantly lower photosynthetic performance under heat stress (compared to all other populations after 60 min at 24 °C, Figure 2e; and compared to the Norwegian population under 36 °C Figure 2m,n,o) suggests that southern-edge populations of *F. serratus* are less heat-stress resistant than populations from its mid-range (supported by Pearson et al., 2009) and northern-edge of distribution. In conclusion, a constitutively high *hsp* expression in Spanish populations of *F. serratus* could reduce their acclimatization potential, thereby increasing sensitivity to further temperature increase.

Instead of indicating chronic thermal stress in northern Spain, the constitutively high *shsp* and *hsp90* expression (Figure 3b,c,e,f) under acclimation conditions may have been induced by cold temperature stress during acclimation (9 °C SST) and thus be an experi-

mental artifact. Average SST in northern Spain is not <12.5 °C during the coldest months (although average SAT drops down to ca. 1 °C, Figure 5a) and 4 weeks at 9 °C might have indeed been stressful. The control temperature of 9 °C was likely within the thermal tolerance range of photosynthetic performance of the Spanish *F. serratus* population, as fluorescence measurements of the Spanish samples did not change significantly from 9 °C to 20 °C (Figure 2a,b,c). Also, *shsp* expression levels were likely unaffected by 9 °C, as they would have decreased over acclimation time from control group 2 (7 weeks acclimation, Figure 3f) to control group 1 (23 weeks acclimation, Figure 3c). This suggests that the constitutive *hsp* upregulation is a chronic stress response of the Spanish population but whether the recorded constitutive *hsp* up-regulation is indeed present in its natural habitat requires measurements of *in situ hsp* expression.

5.3. Where climate change will become too extreme

The climate change scenarios predict that monthly mean temperatures will reach up to 24 °C in Brittany and Spain (Figure 5c), the minimum temperature that inhibited photosynthetic performance in all four populations of *F. serratus* significantly (Figure 2e). Indeed, an inhibitory effect was observed at 22 °C in northern Portugal (Martínez et al., 2012). It is important to realize, however, that our results are based on the physiological responses of adult individuals and juvenile stages are often more susceptible towards environmental change (e.g. Arrontes, 1993; Brawley and Johnson, 1991). The species' physiological response thus confirms the prediction that it will suffer thermal stress and be threatened with extinction along the Spanish and Brittany Atlantic coasts in the next 200 years (Jueterbock et al., 2013). Further exploration of the inter-population variability in heat stress tolerance within the thermal regions will require to investigating the response of more than one population per thermal region.

Contrary to our expectations, the high and unique genetic diversity of the Brittany *F. serratus* population (Coyer et al., 2003; Hoarau et al., 2007) displayed less heat stress resilience compared to the other populations (Figure 2f,l). In contrast, Ehlers et al. (2008) found that genetic diversity increases the heat stress resilience of the eelgrass *Zostera marina*,

with a positive effect on shoot density and on recovery of the entire associated ecosystem. Our findings, however, are based on a sample size of only 6–10 per population, which may be too small to capture the generally high genetic diversity of *F. serratus* in Brittany (Coyer et al., 2003; Hoarau et al., 2007). Disappearance of *F. serratus* from its ancient refugium in Brittany most likely will eradicate the species' center of genetic diversity and adaptability.

6. Conclusions

Photosynthetic performance and cytosolic *hsp* expression varied independently and are likely to measure different physiological processes involved in the heat stress response of a photosynthetic organism. Both stress indicators showed population-specific differences in *F. serratus* with highest resilience in photosynthetic performance found in the species' southern edge population in Spain. Increased thermal tolerance in the Spanish population is likely not adaptive, however, but mediated through constitutively high *hsp* expression levels and may incur an ecological cost of reduced fitness and acclimatization potential to further environmental stressors at the species' southern distributional edge. In the next 200 years, daily summer temperatures are likely to rise above the predicted average temperature of the warmest month ($\geq$24 °C) in the species' glacial refugia of Spain and Brittany. Given the specifically low heat stress resilience in the latter refugium, the species might not have sufficient acclimatization potential to mitigate the predicted extinction south of 45 °latitude and could lose its center of genetic diversity and adaptability. Disappearance of this key species from North-Atlantic rocky shores will precipitate major ecological changes in the entire associated seaweed ecosystem.

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8. Vitae

AJ studies ecological genomics of stress in marine algae as part of his PhD thesis. GH is an evolutionary biologist interested in the genomics of adaptation and hybridization in marine organisms. IS and SK are working in Hoarau's lab on climate change impact and genetic structure of marine organisms. JLO is a molecular ecologist interested in phylogeography and climate change effects on rapid adaptation in fucoids and seagrasses. JAC is a molecular ecologist interested in the phylogeny, phylogeography, and stress response of fucoids and seagrasses. JMOF is a molecular biologist using genomic tools to study muscle growth and the innate immune system in fish.

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The funding agency was not involved in the conduct of the research, preparation of the article, study design, collection, analysis and interpretation of the data or in the decision to submit the article for publication.

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10. Figure captions

Figure 1: Sampling sites where >30 individuals/site were collected in May/June 2011. See supplementary material S8 for precisely mapped locations

Figure 2: Photosynthetic performance under heat stress. Relative change in Pi_{ABS} levels (compared to 9 °C) with bars of 1 standard error, measured from ≥5 *F. serratus* individuals/population during (15 min and 60 min) and after (24 h recovery) exposure to heat stress (20 °C, 24 °C, 28 °C, 32 °C and 36 °C) from each of four populations (Norway, Denmark, Brittany and Spain). A significant difference to the control (zero change in Pi_{ABS}) is indicated by: '*': $p \leq 0.05$. Bars that do not share the same lower case letters indicate significantly different expression levels ($p \leq 0.05$) between the populations at a given time point and temperature.

Figure 3: Relative normalized *hsp* gene expression compared between two control groups of *F. serratus* individuals from four populations (Norway, Denmark, Brittany and Spain) before heat stress exposure; **(a)** control group 1 (23 weeks acclimation at 4 °C, n=6–8) and **(b)** control group 2 (7 weeks acclimation at 4 °C, n=4–6). The control groups did not share the same individuals. The expression quantities, with error bars of 1 standard error, were normalized to the expression levels of two housekeeping genes (*actin* and *eef1*). Bars that do not share the same lower case letters indicate significantly different expression levels ($p \leq 0.05$). Lower case letters are independent between the three *hsp* genes (*hsp70*, *hsp90* and *shsp*). Note the log-scale of the y-axis.

Figure 4: Fold-change in transcriptomic *hsp70*, *hsp90*, and *shsp* gene expression with bars of 1 standard error at 28 °C (n=6–10) and 32 °C (n=4–6) stress (15 min exposure, 60 min exposure and 24 hrs recovery). Changes in gene expression were compared pairwise between *F. serratus* individuals from four populations (Norway, Denmark, Brittany and Spain) within each subplot. Bars that do not share the same lower case letters indicate significant differences. Note the log-scale of the y-axis for *shsp*.

Figure 5: Sea surface temperature (SST) and surface air temperature (SAT) conditions under present day conditions (2000) and predicted for 2100 and 2200 at the four sampling sites of this study (Norway, Denmark, Brittany and Spain; see Figure 1). Yearly averages of monthly mean temperatures are represented by points (SST) or diamonds (SAT). The temperature range (minimum to maximum of monthly means) is represented by continuous (SST) and dashed (SAT) horizontal lines. The short dashed vertical line indicates the minimum temperature (24 °C) at which photosynthetic performance was significantly reduced in all four populations (Figure 2)

11. Tables

Table 1: Primers used for quantitative real-time PCR. The PCR amplification efficiency and the Pearson product-moment correlation coefficient (r^2) of the threshold cycle (Ct) versus log10 cDNA concentration are shown for the two batches of samples that were reverse transcribed together: 1) Spanish, Brittany and Norwegian 28 °C stress samples 2) All 32 °C and Danish 28 °C stress samples. Key: A.n., accession number; *actb*, Beta-actin gene; bp, length of amplicon in basepairs *eef-1*, Eukaryotic elongation factor gene; F, forward; *hsp70*, Heat shock protein 70 gene; *hsp90*, Heat shock protein 90 gene; R, reverse; *shsp*, small heat shock protein 4 gene (*hsp20*).

A.n.	**Gene**	**Primer sequence 5'-3'**	**bp**	**Efficiency**	**r^2**
U11697.1	*actb*	F: AGCGTGGTTACTCCTTCA	105	1.91/2.00	0.988/0.997
		R: CCGTCTTCATCTCCTGGT			
GH700727.1	*eef-1*	F: CCGCTACAAGGAGATCAAGGA	86	1.99/2.13	0.997/0.997
		R: AGATGGGCACGAAGGGAAT			
EU780018.1	*shsp*	F: GACTTCCACGAGACCAACA	75	1.94/2.07	0.999/0.998
		R: CACCTTGATGTCCTCCTTCTT			
EU780017.1	*hsp70*	F: GGGTGCTTATCCAGGTGTA	79	1.93/2.04	0.987/0.998
		R: CCGTCCAGGTTGAACTTG			
EU780016.1	*hsp90*	F: GGTCGCATTCACAGGCTTATC	76	2.02/1.93	0.987/1.000
		R: CGTCCTCTCCGTCGTCTC			

12. Supplementary material - captions

S1: Outliers in photosynthetic performance under heat stress. Relative change of Pi_{Abs} (compared to 9 °C) with bars of 1 standard error, measured from ≥5 *F. serratus* individuals/population during (15 min and 60 min) and after (24 h recovery) exposure to heat stress (20 °C, 24 °C, 28 °C, 32 °C and 36 °C) from each of four populations (Norway, Denmark, Brittany and Spain). Outlier values that were not considered in the data analysis are shown as red dots (not shown: 4.92 change in Pi_{Abs} for Denmark, 24 h recovery from 32 °C stress).

S2: Test results for population and time point effects on photosynthetic performance (normalized Pi_{ABS}). For each stress temperature (20 °C, 24 °C, 28 °C, 32 °C, and 36 °C) test statistics, numerator degrees of freedom (Df) for the central F distribution and corresponding p-values of the test are shown for the two factors Population (Norway, Denmark, Brittany, and Spain) and Duration (0 min or control, 15 min stress, 60 min stress, and 24 h recovery), and the interaction between them (Population:Duration). Significant effects ($p \leq 0.05$) are indicated with "*".

S3: Outliers in relative normalized *hsp* gene expression. Relative normalized *hsp* gene expression compared between two control groups of *F. serratus* individuals from four populations (Norway, Denmark, Brittany and Spain) before heat stress exposure; **(a)** control group 1 (23 weeks acclimation at 4 °C, n=6–8) and **(b)** control group 2 (7 weeks acclimation at 4 °C, n=4–6). The expression quantities, with error bars of 1 standard error, were normalized to the expression levels of two housekeeping genes (*actin* and *eef1*). Outlier values that were not considered in the data analysis are shown as red dots. Note the log-scale of the y-axis.

S4: Outliers in fold-change of transcriptomic gene expression. Fold-change in transcriptomic *hsp70*, *hsp90*, and *shsp* gene expression with bars of 1 standard error at 28 °C (n=6–10) and 32 °C (n=4–6) stress (15 min exposure, 60 min exposure and 24 hrs recovery). Outlier values that were not considered in the data analysis are shown as red dots.

S5: Test results for population and time point effects on the fold change in relative normalized gene expression. For each heat shock protein gene (*hsp70*, *hsp90*, and *shsp*) and stress temperature (20 °C, 24 °C, 28 °C, 32 °C, and 36 °C) test statistics, numerator degrees of freedom (Df) for the central F distribution and corresponding p-values of the test are shown for the two factors Population (Norway, Denmark, Brittany, and Spain) and Duration (0 min or control, 15 min stress, 60 min stress, and 24 h recovery), and the interaction between them (Population:Duration). Significant effects ($p \leq 0.05$) are indicated with "*".

S6: Tests for population differences in photosynthetic performance (Pi_{ABS} values) under control conditions (9 °C) before heat stress exposure (20 °C, 24 °C, 28 °C, 32 °C, and 36 °C). For each pairwise comparison the table shows Tukey contrast effects (Estimator) between populations (Norway, Denmark, Brittany, and Spain) with lower and upper 95% confidence interval limits, the test statistics and p-values. None of the contrasts were significant ($p \leq 0.05$).

S7: ANOVA tables for population and acclimation-time effects on gene expression (normalized expression quantity) of 3 heat shock protein *hsp* genes (*hsp70*, *hsp90*, and *shsp*). For each *hsp* gene, the table shows degrees of freedom (Df), sum of squares (Sum Sq), mean squares (Mean Sq), F-values and p-values for the two factors Population (Norway, Denmark, Brittany, and Spain) and acclimation time (7 weeks and 23 weeks of acclimation), and the interaction between them (Population:Acclimation). Significant effects ($p \leq 0.05$) are indicated with "*".

S8: Precise locations where >30 individuals/site were collected in May/June 2011.