

Some don't like it hot: microhabitat-dependent thermal and water stresses in a trailing edge population

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Summary

1. The distributional limits of species in response to environmental change are usually studied at large temporal and/or geographical scales. However, organismal scale habitat variation can be overlooked when investigating large-scale averages of key factors such as temperature. We examine how microhabitat thermal conditions relate to physiological limits, which may contribute to recent range shifts in an intertidal alga.

2. We defined the onset and maximum temperatures of the heat-shock response (HSR) for a southern edge population of *Fucus vesiculosus*, which has subsequently become extinct. The physiological threshold for resilience (assayed using chlorophyll fluorescence) coincided with declining HSR, determined from the temperature-dependent induction of seven heat-shock protein transcripts.

3. In intertidal habitats, temperature affects physiology directly by controlling body temperature and indirectly through evaporative water loss. We investigated the relationship between the thermal environment and *in situ* molecular HSR at microhabitat scales. Over cm to m scales, four distinct microhabitats were defined in algal patches (canopy surface, patch edge, subcanopy, submerged channels), revealing distinct thermal and water stress environments during low-tide emersion.

4. The *in situ* HSR agreed with estimated tissue temperatures in all but one microhabitat. Remarkably, in the most thermally extreme microhabitat (canopy surface), the HSR was essentially absent in desiccated tissue, providing a potential escape from the cellular metabolic costs of thermal stress.

5. Meteorological records, microenvironmental thermal profiles and HSR data indicate that the maximum HSR is approached or exceeded in hydrated tissue during daytime low tides for much of the year. Furthermore, present-day summer seawater temperatures are sufficient to induce HSR during high-tide immersion, preventing recovery and resulting in continuous HSR during daytime low-tide cycles over the entire summer.

6. HSR in the field matched microhabitat temperatures more closely than local seawater or atmospheric data, suggesting that the impacts of climatic change are best understood at the microhabitat scale, particularly in intertidal areas.

Key-words: algal canopy, climate change, *Fucus vesiculosus*, heat-shock response, HSP gene expression, PSII photochemistry, range edge, thermal limits

Introduction

Studies investigating the effects of environmental change on distribution limits generally focus on large temporal and geographical scales. However, the conditions that organisms experience often depend strongly on local small-scale habitat effects and interactions (Helmuth & Hofmann 2001; Helmuth *et al.* 2006a). Analysis of climatic variation

averaged over large scales can therefore fail to account for extensive variation at scales more relevant to the individuals, species or community in question. This is particularly true in studies linking environmental factors with physiological limits.

A good example is the intertidal zone, where organisms are exposed to both marine and terrestrial environments during tidal cycles. Local variation in extreme and fluctuating environmental conditions may overwhelm large-scale latitudinal temperature gradients (Helmuth *et al.* 2002a,

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2006a). High amplitude temperature shifts at hourly time-scales during low-tide exposure in air or in small pools result in a complex realized thermal environment shaped by local mosaics of abiotic factors, rather than prevailing air or water temperatures (Helmuth & Hofmann 2001; Helmuth 2002b; Seabra *et al.* 2011). In contrast, thermal conditions are comparatively stable during immersion at high tide with relatively little change on daily or even seasonal time-scales (but see Pfister, Wootton & Neufeld 2007). Control of cellular water balance is an additional challenge, particularly for poikilohydric intertidal seaweeds that tolerate rather than prevent cellular water loss. Some algae can lose a large proportion (>90%) of their tissue water over short periods of emersion (Davison & Pearson 1996; Pearson, Lago-Leston & Mota 2009).

Cost-effective, miniaturized and autonomous instrumentation for temperature measurements (Helmuth 2002b; Lima *et al.* 2011) now allow the study of temperature effects at small, ecologically relevant spatial and temporal scales. These developments have been matched by the use of molecular tools to monitor responses to thermal stress in natural populations. The heat-shock response (HSR) is an ancient and ubiquitous cellular response to the potentially lethal accumulation of unfolded protein at elevated temperatures. The sensing of damaged or denatured proteins triggers an evolutionarily conserved response that involves the synthesis of protein chaperones: the highly conserved heat-shock proteins (HSPs). Assessing thermal regimes and relevant physiological responses at local scales may lead to a better understanding of the role of temperature in shaping species distributions. The HSR in natural populations has been studied for relatively few intertidal species occupying distinct thermal niches (Dietz & Somero 1992; Roberts, Hofmann & Somero 1997; Tomanek & Somero 1999). The ecologically relevant components of the stress response include both adaptation to the thermal niche and capacity for acclimation to changing conditions (plasticity).

Concern about the impacts of climate change has renewed interest in the relationship between thermal tolerance limits and species distribution. Such impacts may be higher towards the low latitude range edges, where local habitat effects can become more evident. As edge populations tend to be small and less diverse, local adaptability may be compromised (Pearson, Lago-Leston & Mota 2009) and insufficient to prevent extinction under climatic pressure. In fact, work on intertidal gastropods gives some reason to think that intertidal species may exist near the limits of their thermotolerance, with little capacity for further adaptation of the upper bounds of the HSR (Tomanek & Somero 1999; but see Davenport & Davenport 2005). The somewhat counterintuitive conclusion is that many intertidal species inhabiting highly fluctuating thermal environments have limited acclimatory plasticity and may therefore be at particular risk from climate change (Tomanek 2010).

The trailing edge (southern) distributional boundary for several North Atlantic species is found along SW Iberia. Range contractions and local extinctions in the region have

recently been reported for several ecosystem-structuring marine species (Diekmann & Serrão 2012; Assis *et al.* 2013; Cunha, Assis & Serrão 2013; Nicastro *et al.* 2013). Such effects have been particularly striking for the brown alga *Fucus vesiculosus* L. (Nicastro *et al.* 2013), a major model in studies of intertidal stress (Wahl *et al.* 2011), that at its southern limit occurs exclusively inside estuaries and coastal lagoons. Inside a coastal lagoon in southern Portugal, the species occurred on mudflats as patches that expanded during winter and shrank during summer (Pearson & Serrão, pers. obs.), suggesting seasonally intense environmental stress. Since this study was conducted (in 2008), the formerly extensive patches on intertidal flats have disappeared. Indeed, a dramatic range contraction of 11° latitude over the last 30 years resulted in the local extinction of most southern populations (Nicastro *et al.* 2013).

In this study, we investigated microhabitat variation in realized thermal conditions, hydration status and the molecular heat-shock response in a regressing southern edge population of *F. vesiculosus*, which is now locally extinct (Nicastro *et al.* 2013). Historical climate data are incorporated to develop hypotheses concerning local trends and the biological causes underlying population decline. Conditions measured within each microhabitat diverged considerably from local seawater or atmospheric parameters and matched more closely physiological status under laboratory conditions. Our data suggest that processes mediating responses to environmental change may often only be understood by conducting studies at the relevant organismal scales.

Material and methods

MODEL SPECIES AND STUDY SITE

Fucus vesiculosus plays key ecological roles along the European Atlantic, where it has a mid-high intertidal distribution correlated with stress tolerance limits to temperature and desiccation (Dring & Brown 1982; Pearson, Lago-Leston & Mota 2009; Zardi *et al.* 2011).

This study was conducted in 2008 on a southern edge population in southern Portugal (Ria Formosa coastal lagoon, 37°00'40"N, 7°59'25"W; Fig. S1 in Supporting Information). There, *F. vesiculosus* formed patches on tidal mudflats across a very narrow vertical range (ca. 1.2–2.3 m above ELWS). At the lower vertical limit, individuals occurred also in tidal drainage channels. Sexual reproduction was reduced or absent, with persistence due only to vegetative growth. Although it had been recorded for decades in the Ria Formosa (Ardre 1970 and references therein), in the year following this study, patches became locally extinct and have not recovered. The current southern limit is near Lisbon, except for a remaining site near Cadiz, and unattached vegetative fragments that can still be found entangled in high intertidal *Spartina* in the Ria Formosa (Nicastro *et al.* 2013).

LABORATORY HSR AND STRESS RESILIENCE EXPERIMENTS

Individuals of *F. vesiculosus* were collected in April and July 2008 and acclimated for 4 weeks in 10-L tanks of aerated and recirculating glass fibre-filtered natural seawater at 15 °C under a photosynthetic photon flux density (PPFD) of 25–50

$\mu\text{mol m}^{-2} \text{ s}^{-1}$ for either 10 or 14 h per day (spring and summer experiments, respectively). Thermal stress resilience assays were carried out by exposing acclimated apical tissue ($n = 10$ individuals) to heat shock (HS) for 3 h at 24, 28, 32 and 36 °C (± 0.5 °C) in thermostatically controlled water baths with filtered recirculating seawater. To more closely simulate low-tide field conditions, high PPFD (250–300 $\mu\text{mol m}^{-2} \text{ s}^{-1}$) was provided by sodium vapour lamps. The final temperature was reached by ramping through sequential 15-min transfers between water baths. Controls were manipulated as for the HS treatments, but were maintained under acclimation temperature and irradiance conditions. After HS, algae were allowed to recover under acclimation conditions for 24 h.

Resilience to HS was assayed 24 h following stress exposure using chlorophyll fluorescence (FMS2, Hansatech Instruments). The ratio of photochemical quenching (F_v) to total fluorescence emission from closed PSII reaction centres (F_m) is directly proportional to the quantum efficiency of PSII photochemistry with reductions below maximal values (0.7–0.8 in brown algae) providing a rapid and sensitive indicator of physiological stress. The maximum quantum yield of PSII (F_v/F_m) was determined after 5 min dark adaptation in order to estimate photodamage. While short-term (min) reductions in F_v/F_m can result from physiological adjustments (e.g. dissipation of excess light energy as heat – non-photochemical quenching), the longer-term (24 h) effects reported here can mainly be attributed to photodamaged PSII reaction centres (Maxwell & Johnson 2000).

Samples for RT-qPCR were taken at the end of the HS exposure, and following 24 h recovery, by flash-freezing 5–6 apical tips from $n = 3$ individuals in LN₂. Samples were then stored at –80 °C prior to lyophilization and RNA extraction.

Thermal limits were characterized from the induction profiles of the seven target heat-shock transcripts, using three parameters: T_{on} (onset of HSR induction, the lowest temperature at which a response is observed), T_{peak} (peak induction, the temperature at which the maximal response is observed) and T_{off} (a temperature too high to sustain the HSR, above which no response is observed).

AIR AND SEAWATER TEMPERATURES

Coastal sea surface temperature (SST) warming data were obtained from <http://www.coastalwarming.com> (Lima & Wetthey 2012), based on NOAA data from 1982 to 2011. Seawater temperatures inside the Ria Formosa (ca. 2.5 m below mean low tide) for winter and summer/autumn 2012 were obtained by datalogger (iButtons®; Maxim Integrated Products, Dallas Semiconductor, San Jose, CA, USA) readings at 60 min intervals.

Daily air temperatures from Jan 1973 to Sep 2012 were obtained from weather station 85540 (LPFR) at Faro Airport (37°01'N, 07°58'W) and used to compare seasonal temperature profiles and anomalies. Monthly means were used to fit linear models to determine decadal rates of temperature change.

IN SITU MEASUREMENTS AND SAMPLING

We defined four *F. vesiculosus* microhabitats in the Ria Formosa: 1) 'Channel' = lower shore individuals that remain fully hydrated on all but the most extreme spring low tides, 2) 'Top' = top-canopy of high shore patches exposed to rapid desiccation at low tide, 3) 'Bottom' = individuals lying beneath the canopy surface and 4) 'Edge' = fronds at the periphery of patches lying in contact with moist sediment during low tide. Thermal variation in tissue temperature from different microhabitats was estimated using temperature dataloggers (iButtons®).

Thermal profiles in *F. vesiculosus* patches/channel were recorded every 1 min at low tide on January 28, March 14, Jun 25

and August 6, 2008, on clear and sunny days with a daytime low tide at 12:00–13:00 h. In the channels, loggers ($n = 3$) were placed inside protective brass casing (see Pearson, Lago-Leston & Mota 2009) at the mud/water interface adjacent to the algae that remained immersed. For the other microhabitats, loggers (three per habitat) were lightly sealed with silicon grease, wrapped in white teflon tape and attached with wire clips to the underside of thalli.

In August, apical tips ($n = 5$) were collected in each microhabitat at mid-low tide (13:40 h) and at the end of low tide (16:00 h) for tissue water content estimation. Residual surface water was removed by blotting. Each tip was placed in a pre-weighed vial with 2 mL filtered seawater, re-weighed to obtain the initial weight (IW) of the tissue when sampled. This, together with the hydrated (FW) and dry weights (DW; after drying at 60 °C for 24 h), allows the calculation of the tissue water content (TWC) at the time of collection from:

$$\text{TWC}(\%) = [(IW - DW)/(FW - DW)] \times 100$$

Samples for analysis of the HSR were taken at 1) the onset of emersion, 2) mid-low tide and 3) immediately prior to re-immersion. At each time, 12–15 apical tips (ca. 3 cm) per microhabitat were selected from three patches separated by ≥ 5 m, immediately flash-frozen in LN₂ in the field, and stored at –80 °C.

RNA EXTRACTION AND QPCR

Total RNA was extracted from lyophilized tissue from 30 laboratory HS samples (May experiment; 3 replicates \times 5 temperatures \times 2 times) and from 72 field samples (3 replicates \times 4 microhabitats \times 3 tide times \times 2 months) following Pearson *et al.* (2006), DNase treated and further purified with RNeasy Mini Kit (QIAGEN).

Total RNA was reverse-transcribed with SuperScript III RT (Invitrogen, Carlsbad, CA, USA) and oligo-dT, with an extended synthesis of 1 h at 50 °C followed by 50 min at 55 °C, in two independent (2 μg) reactions for field material, or a single (4 μg) reaction for laboratory HS samples. Additional information on target HSP transcripts and reference genes is given in Table S1 (Supporting Information). qPCRs were performed in triplicate with PerfeCta Fastmix for iQ (Quanta biosciences, Gaithersburg, MD, USA) on an iCycler iQ Detection System (Bio-Rad, Hercules, CA, USA) and analysed using iQ5 software (Bio-Rad). Normalized expression values (geometric mean of the three reference genes) were analysed using the PERMANOVA module in Primer 5.

Results

LABORATORY HSR AND PHYSIOLOGICAL RESILIENCE

Physiological data (F_v/F_m) from Spring and Summer HS experiments were pooled after the analyses indicated no effect of sampling time (PERMANOVA, $P > 0.914$; see Table S2 in Supporting Information). After recovery from temperatures between 24 and 32 °C, F_v/F_m was only slightly (but significantly) lower than in controls at 15 °C (Fig. 1a). In contrast, the effect of 3 h exposure at 36 °C was much greater, indicating that HS resilience was significantly impaired between 32 °C and 36 °C (Table S2 in Supporting Information).

Short-term (3 h) exposure of *Fucus vesiculosus* to HS between 24 and 36 °C induced a HSR in all seven genes

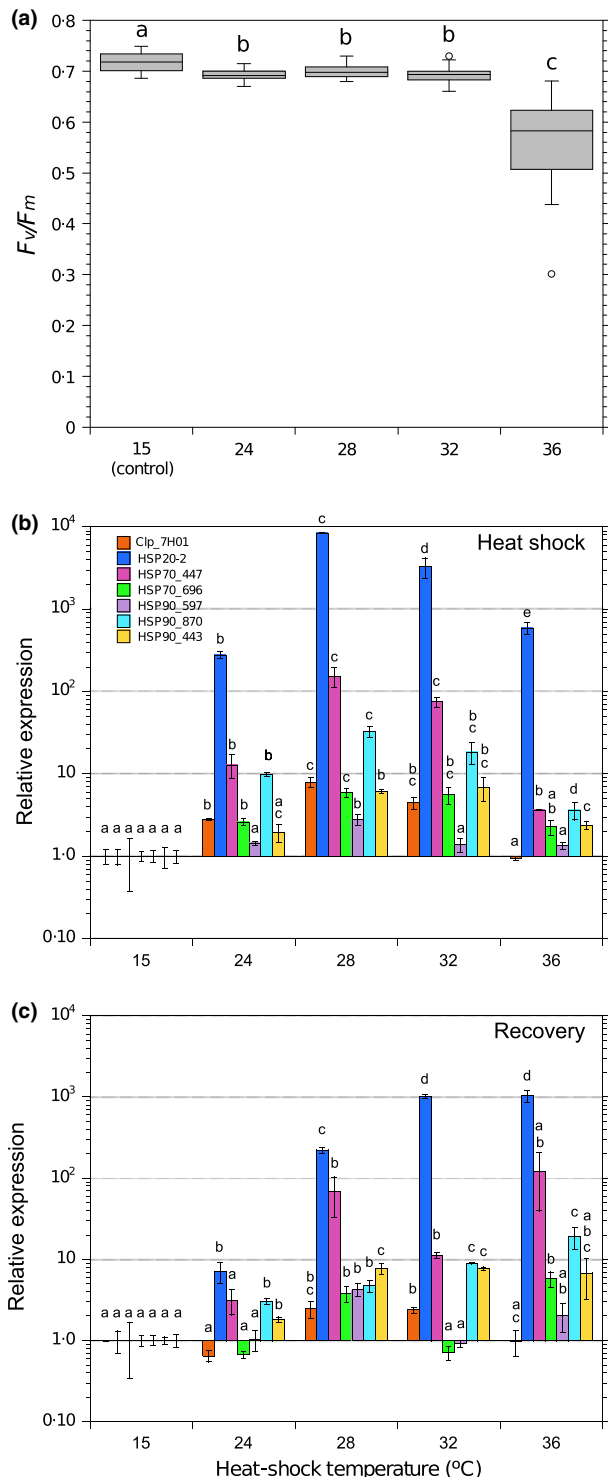


Fig. 1. *Fucus vesiculosus* response after 3 h of heat-shock at 24, 28, 32 and 36 °C and following 24 h recovery. a) F_v/F_m following 24 h recovery from 3 h HS at 24, 28, 32 and 36 °C. Controls were at 15 °C, $n = 20$ individuals, \pm SE. b) Gene expression for seven Hsp transcripts following 3 h HS at the same temperatures as in a). c) Gene expression for the same seven transcripts after 24 h recovery in seawater at 15 °C. Values for b) and c) are normalized (to controls at 15 °C) relative expression values of three individuals, \pm SE. Different letters within each gene indicate significantly different means. (See Tables S2 and S3 in Supporting Information for PERMANOVA and post hoc tests).

(Fig. 1b). Maximum induction across genes ranged between ca. threefold (HSP90_597) and >8000-fold (HSP20-2). The small HSP family chaperone HSP20-2 had the greatest dynamic range, as shown previously (Pearson, Lago-Leston & Mota 2009; Lago-Leston *et al.* 2010; Pearson *et al.* 2010). Peak expression levels were observed at 28 °C for all transcripts, remaining equally high or declining slightly at 32 °C. Expression declined significantly for all genes at the highest temperature tested (36 °C), with no significant induction for three transcripts (see Table S3 in Supporting Information).

After 24 h recovery from 24 °C, all but three transcripts (HSP20-2, HSP90_870 and HSP90_443) had returned to control levels (Fig. 1c). However, expression was still elevated after recovery from exposure to higher temperatures (28–36 °C). After exposure to 36 °C, overall expression was higher after 24 h recovery than immediately post-stress (c.f. Fig. 1b, c). A reduced capacity to mount a HSR at 36 °C and maintenance of high expression levels in the recovery phase correlate with reduced physiological resilience at this temperature (Fig. 1a–c).

MICROHABITAT TEMPERATURE PROFILES

Maximum air temperatures on sampling dates varied from 18.0–22.0 °C (January and March), to 28.0–30.6 °C (August and June; Fig. 2a). Profiles in the four microhabitats were taken on days with similar midday low tides, when algae were emersed between 10:00 and 11:00 h (Fig. 2b). At the onset of low-tide emersion, water temperatures in the channel varied from 14 °C in January, 19 °C in March, to ca. 25 °C in June–August (Fig. 2c–f).

In January (Fig. 2c), water was at ca. 14 °C and fronds peaked ca. 4 °C above maximum air temperature at the canopy surface (Top; 22.2 °C), followed by the Edge (20.8 °C), Channel (20 °C) and the marginally cooler sub-canopy (Bottom; 19 °C). Cloudy conditions in March resulted in temporal variation of up to 5 °C in the Top, while the Bottom (18–19 °C) remained below maximum air temperature (22 °C; Fig. 2d).

Tissue temperatures in June were above 25 °C at the onset of emersion, reaching >35 °C at the Edge of the canopy by mid-afternoon (Fig. 2e). Subcanopy Bottom temperatures increased less, but were still >30 °C for ca. 3 h (Fig. 2e). As in June, August temperatures were >10 °C higher than in January (Fig. 2f). The most extreme microhabitat was the Top, where TM reached 36.7 °C; temperatures of ≥ 35 °C between 14:00 and 15:30 were interrupted only by passing light cloud cover (asterisks, Fig. 2f). Under the canopy was ca. 5 °C lower than the Top (TM = 31.2 °C vs. 36.7 °C on a cm scale) and also increased at a lower rate, reaching 30 °C later and cooling within an hour. In contrast, both the Top and Edge remained at >30 °C for ca. 4.5 h. Similarly, Channel water reached >30 °C for 4 h (Fig. 2f).

Algae at the canopy surface (Top) were severely desiccated by mid-low tide (TWC = $8.5 \pm 0.8\%$, Fig. 2f), while

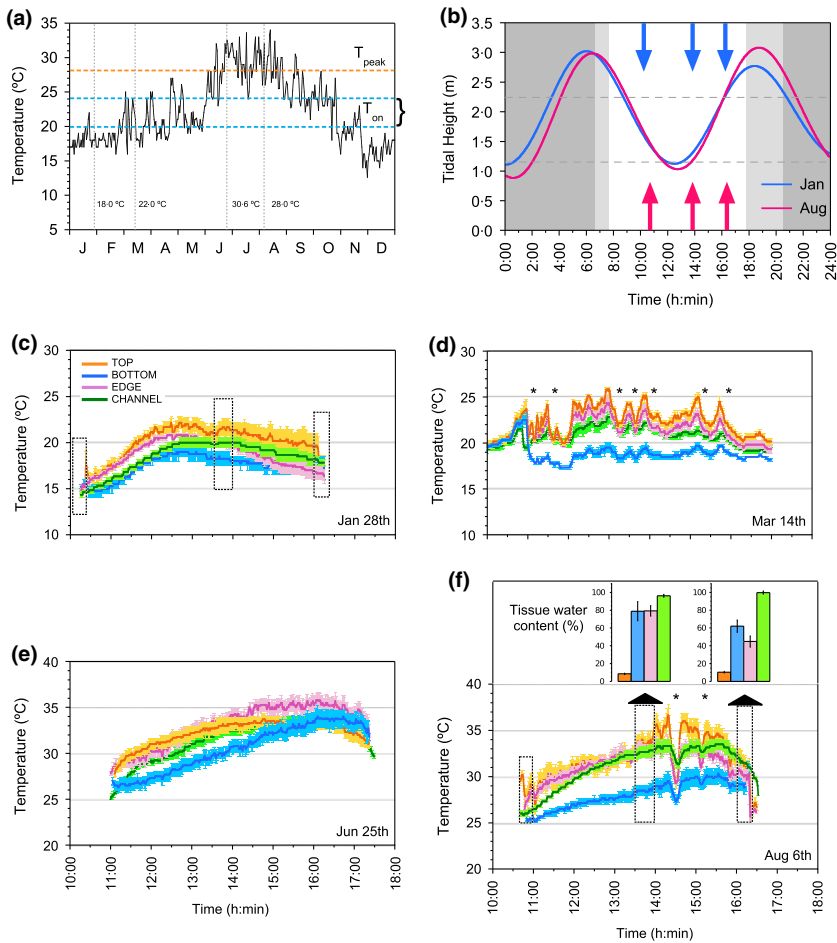


Fig. 2. Site maximum air temperatures, tidal cycle and microhabitat temperatures. a) Daily maximum air temperatures (TM; Faro Airport, 2008). Horizontal broken lines indicate onset of HSR (T_{on}) and peak HSR (T_{peak}). TM for the thermal profiling days are indicated by vertical broken lines. b) Tidal cycles when sampling temperature profiles and for qPCR: coloured arrows show sampling as algae emerged, at mid-low tide, and as they immersed. Dashed lines indicate the vertical distribution of *Fucus vesiculosus* in the Ria Formosa. Shading indicates sunset to sunrise periods. c – f) Microhabitat temperatures (Jan, Mar, Jun, Aug 2008), and tissue water contents (inset bar charts; Aug only). Temperature loggers ($n = 3$, every 1 min) were attached to thalli at the canopy top (orange), bottom (blue) and edge (purple) and in a channel (green), covering the vertical range. Asterisks = light cloud cover. Sampling times for HSR are the dashed boxes (Jan, Aug).

at the other microhabitats they had some refuge from desiccation—Channel algae were fully immersed, Edge algae were in contact with the wet sediment or immersed in small depressions, and Bottom algae were protected from evaporative water loss thermally and by shading. At the end of low tide, Edge and Bottom algae still retained $44.8 \pm 6.8\%$ and $62.1 \pm 6.9\%$ TWC, respectively (Fig. 2f, inset bar plots).

HS GENE EXPRESSION PROFILES IN NATURAL STANDS OF *F. VESICULOSUS*

In January and August, the expression of Hsp transcripts in field samples under known temperatures at low tide (Fig. 2) can be compared with laboratory baseline HSR and physiological resilience (Fig. 1). The data plotted for each transcript (see Fig. 3 and Tables S4 and S5 in Supporting Information) are normalized to the average initial values for January across all microhabitats, thus allowing comparison of spatial and temporal variation. Transcriptional responses in January were small in all microhabitats, concordant with a temperature range of $14.6\text{--}21.4\text{ }^{\circ}\text{C}$ (Fig. 3a–d). Although the highest temperatures were observed at the Top, no significant HSR occurred there. However, a mild HSR was clearly evi-

dent in the Channel microhabitat, with a small but significant induction of five transcripts at mid-low tide (Fig. 3d).

The over-expression of HSP20-2 transcripts in August compared with January (ca. 100-fold) was striking in all microhabitats at the onset of low tide (Fig. 3). These results suggest that significant HS expression had either already been induced in seawater (ca. $26\text{ }^{\circ}\text{C}$) prior to the first sampling period, or that transcript levels remained elevated due to previous stress cycles.

Field and laboratory HSRs agree well for algae under similar hydration status and temperature: c.f. 3 h laboratory HSR at $32\text{ }^{\circ}\text{C}$ (Fig. 1b) vs. Channel HSR at mid-low tide in August (ca. 2.5 h between $28\text{--}33\text{ }^{\circ}\text{C}$; Figs 2f and 3h). The Loglinear relationship between field expression values for HSP20_2 (the transcript with the highest dynamic range) and temperature was highly significant across samples for January and August (Fig. 4). Furthermore, estimates of T_{on} for the HSP20_2 HSR transcript vary little when combined January and August data, or solely August data are considered (ca. $21\text{--}23\text{ }^{\circ}\text{C}$), showing little evidence for seasonal acclimation (plasticity).

There was a clear mismatch between temperature and HS gene expression in the Top microhabitat linked with

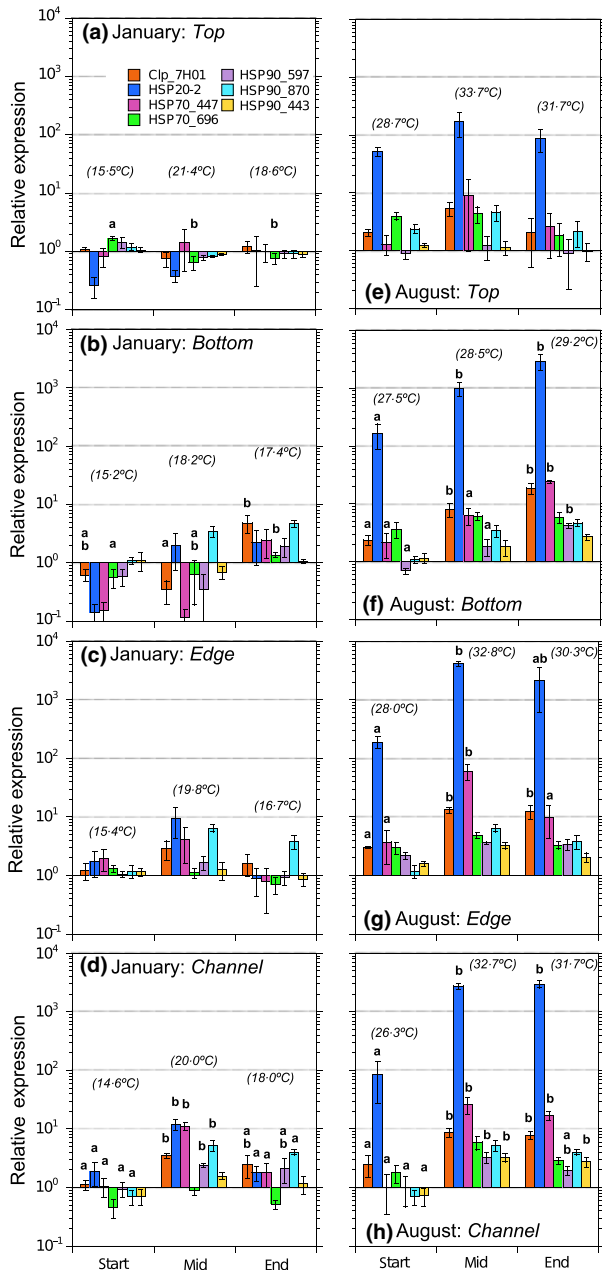


Fig. 3. Gene expression for seven Hsp transcripts in the field microhabitats in January (a–d) and August (e–h) 2008. *Fucus vesiculosus* samples were taken at the onset of low tide (=start), mid-low tide (=mid) and prior to re-immersion (=end). Values are relative expression assayed by RT-qPCR, normalized to the mean of initial values of the transcript over all microhabitats in January ($n = 3$ individuals, \pm SE). Values with the same letter within a gene and month are not significantly different. Italics show the temperature at the time of sampling in each microhabitat and phase of the low tide. (See Tables S4 and S5 in Supporting Information for PERMANOVA and post hoc tests).

desiccation (Fig. 3a, e). No inducible HSR occurred at the canopy surface either in August (36 °C) or January (22 °C), while seawater at 20 °C already elicited a mild HSR in hydrated algae (Fig. 3d). The canopy surface rapidly desiccated to <10% TWC in the first hours of emersion, resulting in transcriptional arrest.

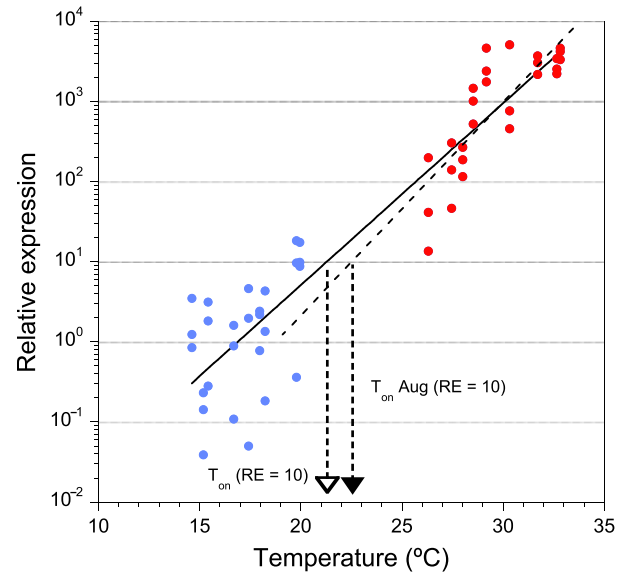


Fig. 4. Relationship between temperature and HSP20_2 expression for January and August field samples. Relative expression (RE) of sHsp (HSP20_2) in field-collected samples of *Fucus vesiculosus* from January (blue) and August (red) in relation to tissue temperature. Values are for biological replicates taken from all microhabitats except the canopy surface (i.e. edge, bottom and channel), sampled at the start, mid and end of low tide (as shown in Fig. 2b); $n = 54$ (January + August) or 27 (August only). A linear regression fitted to $\text{Log}_{10}(\text{RE})$ gives $\text{RE} = -3.8398 + 0.22719(T^{\circ}\text{C})$ $R^2 = 0.88393$ (solid black line). For August data only, the regression gives $-4.9961 + 0.2659(T^{\circ}\text{C})$ $R^2 = 0.6728$ (broken black line). At $\text{RE} = 10$, the estimates for HSR T_{on} are 21.3 °C and 22.5 °C, respectively (broken open and filled arrows).

CURRENT TEMPERATURE REGIMES AND REGIONAL WARMING TRENDS

Maximum daily air and hourly seawater temperatures in the Ria Formosa in 2012 provide a picture of an extreme thermal environment for *F. vesiculosus* (Fig. 5). Maximum air temperature began to exceed field-estimated T_{on} for the HSR in fully hydrated algae (ca. 20 °C; Fig. 3d) in March and mostly exceeded the 10-fold induction of a sHsp transcript at 21–23 °C (Fig. 4) between May and Sep. Seawater temperatures exceed these values for the entire summer period (except during spring tides tidal flushing). So *F. vesiculosus* would have only intermittent refuge from HS on spring tides and would be exposed to HS each daytime low tide. The frequency of such events is clustered, since daytime low tides between 11:00 and 15:00 h (when solar/thermal exposure is highest) occur during five or six consecutive days, twice monthly.

Southern Portuguese coastal waters have experienced significant warming over the last four decades; SST adjacent to Faro (36°52'50"N, 7°52'50"W) indicates mean warming rates of 0.29 °C/decade (Lima & Wetthey 2012; Nicastro *et al.* 2013). Warming is mainly seen from Apr to Jun and Oct to Jan (Table 1), resulting in earlier warming (based on changes exceeding 75% of SSTs) of ca. 10 Julian

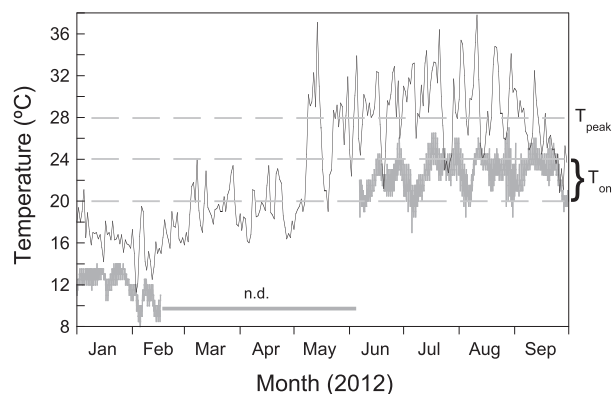


Fig. 5. Air and seawater temperatures in the Ria Formosa in 2012. Maximum daily air temperatures for 2012 from Faro Airport weather station (black line) and SW temperatures within the Ria Formosa (ca. 2–5 m below MLT, see Fig. S1; grey line). 2012 SW temperatures were measured hourly by datalogger. For reference, the HSR T_{on} range and T_{peak} value estimated from laboratory and field data are shown as horizontal broken lines.

days/year, and with between 11 and 15 more days/year warmer than 95% of SSTs.

Monthly minimum air temperatures for Faro are increasing for all but the winter months, at around 1 °C/decade, with lower rates for mean and maximum temperature, both significant in spring (Table 1). Decadal means for 1973–2011 show a clear increasing trend that is particularly marked in the spring and early summer, while the trend in maximum air temperatures is less dramatic (see Fig. S2 in Supporting Information). Analysis of daily temperature anomalies in 1973–2011 shows a similar steadily mean increase, with the largest shifts in the spring and early summer (see Fig. S3 in Supporting Information).

Discussion

This study presents the most comprehensive characterization yet of the heat-shock response (HSR) in an ecosys-

tem-structuring alga. The data were recorded from a southern edge population immediately prior to local extinction, with microenvironmental thermal profiles, together with molecular and physiological data on HSR, revealing the common occurrence of non-permissive temperatures in natural algal patches.

We first established the temperature dependence of the HSR for the expression of seven chaperones, encompassing the range of environmentally relevant temperatures up to levels that severely affect physiological resilience. Secondly, *in situ* thermal profiles and HSR indicated that local (microhabitat) scale tissue temperatures at or above T_{peak} and seawater temperatures around T_{on} , resulted in extreme thermal loads throughout the entire summer period, likely resulting in a semi-permanent, or chronic HSR. Thirdly, we present the first molecular evidence that desiccation arrests or prevents the HSR. As a consequence, the canopy surface in natural populations did not display the same temperature-dependent HSR kinetics as that shown in hydrated tissue, despite reaching the highest temperatures. While other causes have not been excluded, local and regional warming trends over the last 40 years are consistent with steadily increasing thermal stress loads that may have contributed to population decline and range contraction at the trailing southern edge (Nicastro *et al.* 2013). A recent study combining biogeographic patterns of genetic diversity with species distributional modelling identified both increasing sea surface and air temperatures as explanatory factors (Assis *et al.* 2014).

THE HSR OF *FUCUS VESICULOSUS*

The main features of the *F. vesiculosus* HSR were established in laboratory experiments with a suite of seven HSP transcripts. The onset temperature (T_{on}) was below 24 °C (the lowest temperature tested), and T_{peak} occurred at 28 °C, with induction and peak expression in close agreement for all the transcripts. Importantly, the decline in

Table 1. Rates of change in sea surface temperature (SST) and mean minimum (Tm), average (T) and maximum (TM) air temperatures in the Faro region. Based on data from 1982 to 2010 for seawater (Lima & Wetthey 2012) and 1973 to 2011 for air temperatures

Season	SST*			Tm		T			TM			
	Month	P-value	Rate (°C/decade)	R^2	P-value	Rate (°C/decade)	R^2	P-value	Rate (°C/decade)	R^2	P-value	Rate (°C/decade)
Winter	Jan	<0.05	0.21	0.098	n.s.	0.42	0.079	n.s.	0.23	0.007	n.s.	0.05
	Feb	n.s.	0.20	0.070	n.s.	0.41	0.074	n.s.	0.27	0.029	n.s.	0.15
Spring	Mar	n.s.	0.10	0.447	<0.0001	0.99	0.373	<0.0001	0.58	0.036	n.s.	0.18
	Apr	<0.05	0.31	0.515	<0.0001	1.12	0.409	<0.0001	0.73	0.169	0.009	0.44
Summer	May	<0.05	0.37	0.616	<0.0001	1.07	0.369	<0.0001	0.69	0.109	0.04	0.35
	Jun	<0.05	0.61	0.496	<0.0001	0.88	0.303	0.0003	0.68	0.185	0.006	0.57
Autumn	Jul	n.s.	0.33	0.386	<0.0001	0.71	0.129	0.025	0.34	0.060	n.s.	0.24
	Aug	n.s.	0.38	0.435	<0.0001	0.77	0.135	0.021	0.33	0.000	n.s.	0.01
Winter	Sep	n.s.	0.09	0.234	0.002	0.51	0.033	n.s.	0.16	0.063	n.s.	-0.25
	Oct	<0.05	0.49	0.335	0.0001	0.80	0.275	0.001	0.63	0.050	n.s.	0.22
Winter	Nov	<0.05	0.33	0.033	n.s.	0.24	0.057	n.s.	0.25	0.000	n.s.	0.01
	Dec	<0.05	0.23	0.011	n.s.	0.14	0.039	n.s.	0.19	0.000	n.s.	0.01

*Station 11_8152: 36°52'50"N, 07°52'05"W.

capacity to mount a HSR at 36 °C coincided with a loss of resilience in PSII photochemistry, implying fitness costs associated with enhanced rates of protein turnover and repair. Similar induction patterns were reported in intertidal brown algae (although responses differ in subtidal populations) for a single Hsp70 gene (Henkel, Kawai & Hofmann 2009) and in the lower shore *Fucus serratus*, for three Hsp genes (Jueterbock *et al.* 2013). At temperatures that exceed T_{peak} and impair photosynthetic physiology (36 °C), HSP expression was greater after recovery than immediately post-stress. Extensive repair of misfolded and aggregated proteins therefore appears to be required at least 24 h after a return to growth temperatures, implying non-trivial metabolic costs and impacts on energy allocation for normal metabolism and growth.

DOES MICROHABITAT VARIATION MODERATE THERMAL STRESS?

We hypothesized that microhabitat variation provides distinct local environmental conditions, which should result in variable stress loads during emersion. In this case, it is interesting to note that (with the exception of channels) these microhabitats arise solely from the patch structure created by *F. vesiculosus*, rather than from intrinsic habitat heterogeneity (e.g. shading due to aspect or crevices on rocky intertidal shores). More specifically, we hypothesized that the top and edges of patches would be more thermally stressful than shaded lower canopy or channels. In fact, the situation was quite complex. The bottom, or subcanopy, was consistently the most thermally benign microhabitat, with the lowest warming rate and generally the lowest maximum temperature attained (but see Fig. 2e). Algal canopies are generally considered to provide relatively cool and moist conditions favourable for growth (Brawley & Johnson 1991; Davison, Johnson & Brawley 1993), and this was reflected in delayed and/or reduced HSR. However, even here a severe HSR was observed in summer. The similarity in thermal conditions and HSR in patch edges and channels was striking, and although channels warmed at a slightly lower rate, our data show that they did not provide a significant escape from thermal stress.

Contrary to our predictions, we found that the hottest and driest microhabitat, the top of the canopy, may actually be the most benign for a poikilohydric alga like *F. vesiculosus*. The occurrence of rapid and severe desiccation prevents any transcriptional response to thermal stress and may have a protective role, by keeping fronds in a metabolically inactive state. Desiccation is a well-known survival strategy not only for resting stages such as seeds and spores, but for a variety of adult taxa as well, in which organisms can enter a remarkable ametabolic state known as anhydrobiosis. In most organisms, the desiccated state increases thermotolerance (Alpert & Oliver 2002), and this is a confirmed but little-explored feature of some intertidal macroalgae (Hunt & Denny 2008). We tentatively suggest that poikilohydry may be an alternative strategy that

allows individuals to escape the energetic costs of mounting a HSR during peak stress periods. If so, it would be a particularly important adaptation to life near the southern range edge. Additional work is required to elucidate the potential benefits of this strategy in the long term, to determine the metabolic costs, not only of desiccation but also of the subsequent rehydration/recovery process, and compare them to the costs associated with a HSR in the hydrated state.

THERMAL CONDITIONS AT THE SOUTHERN EDGE; A CHRONIC HEAT STRESS ENVIRONMENT

Contrasting winter and summer thermal conditions were reflected in large differences in HSP induction. Optimal warming conditions in January (full sun, air temperature of 18 °C) induced a small but significant HSR in submersed algae warmed to 20 °C, but recovery to initial conditions had occurred by the end of low tide. The fact that we could detect a minor HSR close to 20 °C in the field does, however, support what we saw in laboratory trials, in which a clear HSR was already evident after exposure to 24 °C and suggests a T_{on} nearer to 20 °C than 24 °C. The current average winter maximum air temperature remains below 18 °C in the Ria Formosa, indicating that thermal stress during winter months is unlikely to have significant effects on metabolism and growth.

In contrast, during equivalent low-tide periods in summer, levels and frequency of heat-shock exposure were extreme, based on the observed parameters of the *F. vesiculosus* HSR. Tissue temperatures were consistently above maximum air temperatures, and therefore likely to surpass the T_{peak} throughout summer when algae are emersed during daytime low tides, resulting in a widespread and prolonged HSR in hydrated algae. Even under the moderate August sampling conditions (maximum air temperature 28.0 °C), tissue temperatures in all microhabitats (31–36 °C) exceeded T_{peak} during the low tide sampled. Repeated exposure to such temperatures and induction of HSR over five or six consecutive days during daytime low tides would impose large metabolic costs. High mortality in response to repeated heat-shock exposures has been documented in intertidal mussels (Jones, Mieszkowska & Wethey 2009). Multiple sequential exposures to high temperatures likely decrease thermal tolerance thresholds (Jones, Mieszkowska & Wethey 2009; Sorte, Jones & Miller 2011), since individuals are still repairing accumulated damage from previous exposures.

The HSR has been characterized in very few intertidal species. Reported T_{on} was higher in an intertidal snail (27 °C) than the 20–24 °C we determined here for *F. vesiculosus* (Tomanek & Somero 1999), but in a similar range or even lower for low intertidal to subtidal kelp species (Henkel & Hofmann 2008; Henkel, Kawai & Hofmann 2009). An important question in seasonally varying thermal environments is the degree of plasticity shown by the HSR. Somewhat counterintuitively, however, it appears

that the thermal variability of intertidal habitats may constrain the evolution of significant plasticity. Intertidal organisms generally live near to their thermal limits, frequently inducing a HSR in nature as part of their life-history strategy, and in contrast to subtidal organisms that rarely experience temperatures near to their observed T_{on} (Tomanek 2010). While we did not directly address this question, the apparently small seasonal variation in T_{on} we observed in sHSP induction in the field (Fig. 4) supports this idea.

An additional and chronic stress for southern populations is predicted by seawater temperatures during immersion that are non-permissive for HS recovery. In the shallow Ria Formosa, seawater temperatures of 20–26 °C prevail during summer, resulting in a semi-constant T_{on} for the HSR. This is the likely cause of the elevated sHSP expression observed in August compared with January at the onset of low tide (Fig. 3). South of Lat 41 °38'N (i.e. northern Portugal), *F. vesiculosus* occurs exclusively in estuaries and embayments where decadal scale rising air temperatures also increase warming of local semi-enclosed water masses. In contrast, in northern Portugal (the southern edge of open coast rocky intertidal populations and more typical habitat for the species) seawater temperatures during immersion do not exceed the estimated T_{on} range of 20–24 °C (Pearson, Lago-Leston & Mota 2009). A detailed understanding of the interaction of seawater and air temperatures on the timing and extent of the HSR, together with direct measures of fitness-related traits, could be powerful in developing predictive niche models for population persistence throughout the (declining) southern range of the species (Assis *et al.* 2014).

Several recent studies have documented the impacts of climate change on intertidal invertebrates and algae (reviewed in Helmuth *et al.* 2006b; Hawkins *et al.* 2009), revealing widespread northern range expansions and some southern range contractions since the 1970s/1980s, coinciding with the steady increase in regional coastal SST. While other factors cannot be excluded, regional sea surface warming trends over recent decades correlate with the local extinction of the Ria Formosa population, as well as other southern edge populations of *F. vesiculosus* (Nicastro *et al.* 2013). Combining microenvironmental measurements with characterization of the HSR shows that these edge populations exist(ed) very near to their thermal limits, and together provide a strong argument that thermal stress plays a significant role in population persistence near the southern (trailing) edge.

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Data accessibility

Data available from the Dryad Digital Repository: <http://doi.org/10.5061/dryad.mr72n> (Mota *et al.* 2014).

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Fig. S1. Map of Portugal showing Ria Formosa. The sampling (yellow arrow) and SW temperature measurements (white arrow) sites are indicated.

Fig. S2. Decadal temperature trends in Ria Formosa. Monthly averages for mean and maximum air temperatures for 1973–2011.

Fig. S3. Average daily mean temperature anomalies in Ria Formosa 1973–2011.

Table S1. Primers for RT-qPCR and PCR reaction efficiencies for laboratory and field-collected samples.

Table S2. PERMANOVA and pairwise post hoc comparisons of F_v/F_m from laboratory HS experiments.

Table S3. PERMANOVA and pairwise post hoc comparisons of HSR after 3 h HS and subsequent recovery.

Table S4. PERMANOVA and pairwise post hoc comparisons of January HSR in four microhabitats.

Table S5. PERMANOVA and pairwise post hoc comparisons of August HSR in four microhabitats.