

Loss of thermal refugia near equatorial range limits

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Abstract

This study examines the importance of thermal refugia along the majority of the geographical range of a key intertidal species (*Patella vulgata* Linnaeus, 1758) on the Atlantic coast of Europe. We asked whether differences between sun-exposed and shaded microhabitats were responsible for differences in physiological stress and ecological performance and examined the availability of refugia near equatorial range limits. Thermal differences between sun-exposed and shaded microhabitats are consistently associated with differences in physiological performance, and the frequency of occurrence of high temperatures is most probably limiting the maximum population densities supported at any given place. Topographical complexity provides thermal refugia throughout most of the distribution range, although towards the equatorial edges the magnitude of the amelioration provided by shaded microhabitats is largely reduced. Importantly, the limiting effects of temperature, rather than being related to latitude, seem to be tightly associated with microsite variability, which therefore is likely to have profound effects on the way local populations (and consequently species) respond to climatic changes.

Keywords: biogeography, heat shock proteins, intertidal, microhabitat, thermal refugia, thermal stress

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Introduction

Climate change is a major threat to global biodiversity, and one of the greatest challenges today is to predict how it will impact natural systems (Pereira *et al.*, 2010). Mechanistic models hold great promise as predictive tools (Kearney & Porter, 2009), but are hard to implement because they require the ability to downscale global climatic patterns into microclimates, and to understand how these, in turn, drive the physiology of the organisms (Huey, 1991; Helmuth *et al.*, 2010). In fact, as body temperatures are neither necessarily correlated with latitude or easily obtained from large-scale climatic data (Helmuth & Hofmann, 2001; Potter *et al.*, 2013; Lathlean *et al.*, 2014; Seabra *et al.*, 2015), the recent development of autonomous biomimetic devices has given researchers long-needed tools to tackle this problem (Helmuth & Hofmann, 2001; Fitzhenry *et al.*, 2004; Lima & Wethey, 2009), allowing them to obtain long-term records of body temperatures over continental scales (e.g., Seabra *et al.*, 2011, 2015).

Rocky intertidal systems are inhabited by species that can be exposed to extreme thermal stress during tide-out emersion (see Helmuth *et al.*, 2006; for a review). Solar radiation is usually the dominant component of the surface energy balance during low tide, causing body temperature variations of up to 30 °C in just a few hours (e.g., Southward, 1958; Helmuth & Hofmann, 2001). Yet, solar radiation does not reach all intertidal organisms in a uniform way. Poleward-facing surfaces receive less direct radiation and thus are cooler than equatorward-facing surfaces (Wethey, 2002; Kearney & Porter, 2009; Miller *et al.*, 2009), and it has been recently shown that temperature differences between sun-exposed and shaded microhabitats are higher than those associated with seasons, latitude or shore level (Seabra *et al.*, 2011). Thus, local geomorphology has the potential to either amplify the deleterious effects of regional climatic conditions or to provide thermal refugia, which may be extremely important for some species, particularly towards their distributional limits (Bennie *et al.*, 2008). It must be acknowledged, however, that different temperatures do not necessarily imply different thermal stress levels. Other factors, such as species-specific variability in physiological tolerance (e.g., Dong *et al.*, 2008), mobility (which may

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allow for behavioural thermoregulation, see Huey *et al.*, 1989; Chapperon & Seuront, 2011; Marshall *et al.*, 2013), or the ability to acclimate (e.g., Meng *et al.*, 2009) are also important as well. In that sense, elevated temperatures are only relevant if they lead to physiological stress (Helmuth *et al.*, 2010). Therefore, the main aims of this study were: (i) to find whether the pervasive temperature differences associated with sun-exposed and shaded microhabitats (Seabra *et al.*, 2011) translated into different levels of physiological stress and (ii) to evaluate if differences in physiological stress between sun-exposed and shaded microhabitats within each shore varied along the geographical distribution of a key intertidal species, in particular towards its equatorial range limit. To that end, we quantified the endogenous levels of heat shock proteins (Hsp) on the intertidal limpet *Patella vulgata* Linnaeus, 1758, collected from both sun-exposed and shaded microhabitats over most of its distribution along the European Atlantic coast. Ecological physiologists have been using Hsps as indicators of thermal stress because their synthesis closely tracks the thermal history of organisms (Hofmann, 2005). These proteins are molecular chaperones that confer thermal tolerance by stabilizing other proteins that would otherwise denature, thus allowing normal cellular functions to continue even at elevated temperatures (Feder & Hofmann, 1999; Hofmann *et al.*, 2002). Hsp can be divided into several families based on their molecular weight (Feder & Hofmann, 1999). Endogenous levels of the Hsp70 family, which includes constitutive and inducible isoforms, have been shown to vary between congeneric species (Tomanek & Sanford, 2003; Dong *et al.*, 2008) and in relation to experimental acclimation in lab and seasonal acclimatization in the field (Buckley *et al.*, 2001). Importantly, Hsp70 expression reflects both the thermal variability associated with different microhabitats and with large-scale environmental gradients across whole distributional ranges (Halpin *et al.*, 2002; Snyder & Rossi, 2004; Sorte & Hofmann, 2005; Sagarin & Somero, 2006).

Patella vulgata is appropriate for this study due to several reasons. It can be abundantly found over the majority of the Atlantic European continental coast south of Norway but reaches its equatorward distribution limit in SW Iberia and is absent from the Bay of Biscay (Fischer-Pi ette & Gaillard, 1959; Christiaens, 1973). This geographical pattern is repeated in a variety of algae and invertebrates (Fischer-Pi ette, 1955; Crisp & Fischer-Pi ette, 1959; Southward *et al.*, 1995), meaning that *P. vulgata* is a good model for a wider range of organisms. In addition, *P. vulgata* is a keystone species whose grazing activities control, to a large extent, local biodiversity and community structure throughout much of its range in Europe (Southward, 1964; Haw-

kins & Hartnoll, 1983; Raffaelli & Hawkins, 1996; Jenkins & Hartnoll, 2001; Jenkins *et al.*, 2005; Coleman *et al.*, 2006). Experimental work has confirmed that changes in the abundance of *P. vulgata* influence the entire community (Hawkins *et al.*, 2008, 2009). Thus, a better understanding of the factors that control the distribution of this species can improve our ability to forecast responses of intertidal ecosystems to climate change.

Material and methods

Microhabitat body temperature

Intertidal temperatures were recorded at 17 wave-exposed shores along the European Atlantic coast, spanning nearly 20° of latitude, from southwest Scotland to south Portugal (Fig. 1c). Data were acquired using robotlimpets, autonomous temperature logging devices mimicking the visual aspect and temperature trajectories of real limpets (Lima & Wethey, 2009). Loggers were deployed following Seabra *et al.* (2011). We sampled temperatures from four distinct combinations of height above the low water mark (mid and high shore) and exposure to sun (shaded and sun-exposed), thus covering the majority of the range of microhabitats occupied by *P. vulgata*. Data were collected continuously between the summers of 2010 and 2014 at a sampling interval of 60 minutes and a resolution of 0.5 °C. For each location, we averaged data from loggers sharing the same orientation in relation to the sun (i.e. sun-exposed and shaded).

Distribution of Patella vulgata

Population densities of *Patella vulgata* were measured during spring low tides in July and August 2012 (see Table 1). At each location, individuals were counted using a total of thirty 30 × 30 cm quadrats haphazardly placed at locations equivalent to the microhabitats where temperature loggers were deployed (half in shaded microhabitats and the other half in sun-exposed microhabitats, throughout the vertical distribution of the species in the mid and high intertidal).

Tissue sampling and western blotting

Patella vulgata were collected at each location, in close proximity to the loggers (<5 m), during the summer of 2012 in all studied shores (see Table 1). We collected eight individuals from shaded microhabitats (north-facing walls and/or crevices) and eight individuals from sun-exposed microhabitats (south-facing rocky outcrops), throughout the range of shore heights in which *P. vulgata* occurs in each location. To minimize potential physiological variation related to endogenous tidal rhythms, collections were made within 30 min of low tide (Tomanek & Sanford, 2003). Animals were immediately frozen on dry ice upon collection and kept at −80 °C until further processing.

Patella vulgata displays a range of homing habits, mostly depending on the geographical region and inclination of

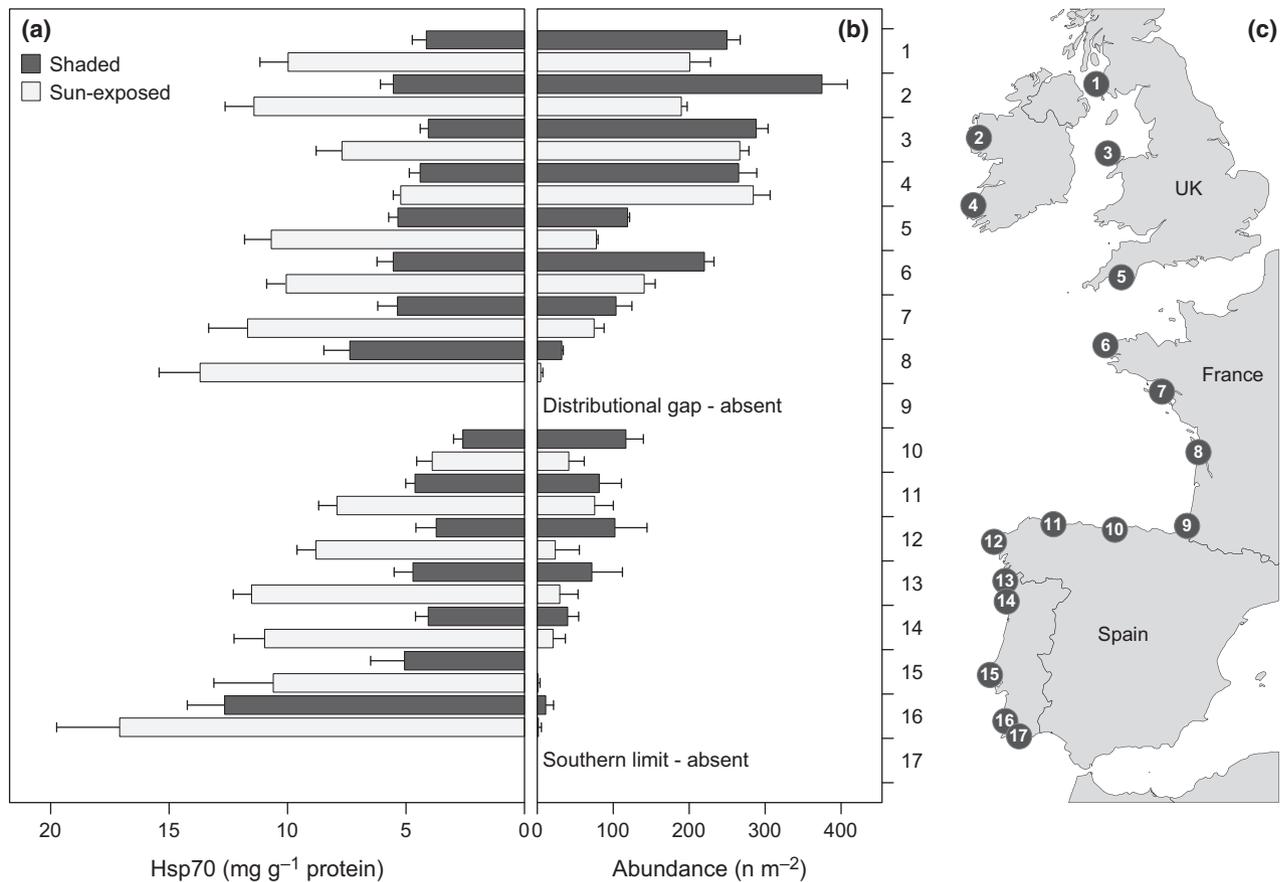


Fig. 1 Hsp70 levels and abundances of *Patella vulgata* along the European Atlantic coast. (a) Mean Hsp70 levels \pm 1 SEM from animals collected in shaded (light grey) and sun-exposed (dark grey) microhabitats ($n = 8$ for all bars). Hsp70 levels were significantly higher in sun-exposed than in shaded microhabitats ($P < 0.01$ after ANOVA). (b) Mean abundances \pm 1 SEM in shaded (light grey) and sun-exposed (dark grey) microhabitats ($n = 15$ for all bars with the exception of location 8 where $n = 10$). (c) Map of the sampled locations (for further details, see Table 1).

rock surfaces. In the middle of its range (UK), it may forage during the day at high tide (Hartnoll & Wright, 1977), at low tide when it is damp (but not raining, Santini *et al.*, 2004) or during night-time low tides (Hawkins & Hartnoll, 1982). At lower latitudes, unless the environment is extremely humid, it moves only during the night, and returns to its home scar during the day, firmly clamping to the rock to reduce desiccation (Branch, 1981; Gray & Naylor, 1996). Thus, throughout the entire species range, individuals stay immobilized during the most thermally stressful part of the day. As part of a separate monitoring programme, 800 individuals of *P. vulgata* were colour-tagged in four locations along the Portuguese coast (400 in shaded and 400 in sun-exposed microhabitats), and their movement patterns registered at a monthly basis for 1 year (2010–2011). All tagged individuals remained faithful to their home scars during the entire duration of the study (F. P. Lima, unpub. data). Consequently, there is high confidence that, prior to collection, individuals used for Hsp70 quantification had been in the same microhabitat where they were collected from (at least at the temporal scales relevant for this study). Likewise, temperatures logged by (static) robolimpets are representative of the

diurnal body temperatures experienced by *Patella* spp. found in their vicinity during low tide.

In the laboratory, approximately 20 mg of tissue were dissected from the foot of each animal and placed in lysis buffer [0.4% Tris-HCl, 2% sodium dodecyl sulphate (SDS), 1% ethylenediaminetetraacetic acid (EDTA), 1% Protease inhibitor cocktail (Thermo Scientific #78444, Rockford, IL, USA), pH 6.8]. Samples were incubated for 5 min at 100 °C and mechanically homogenized at 30 Hz for 5 min using a Retsch MM400 homogenizer. After repeating this procedure two times, homogenates were spun for 15 min at 15 800 g and stored the supernatant at -80 °C until further processing. 10 μ L of each sample were used for total protein concentration determination, using a BCA protein assay kit (Pierce #23225, Rockford, IL, USA). These protein concentrations were later used for loading similar amounts (mass-wise) of sample into the gels.

Samples were taken from the -80 °C freezer, boiled at 100 °C for 5 min and then diluted 1 : 1 in Laemmli sample buffer (Sigma-Aldrich #S3401, St. Louis, MO, USA). Protein separation was carried out by electrophoresis on small format SDS-polyacrylamide gels using Mini-PROTEAN Tetra Cell cast systems, BioRad, Hercules, CA, USA (4% for stacking gels

Table 1 Geographical locations and respective sampling dates

Ref	Location name	Geographical location		Sampling date
1	South Cairn, UK	54°58'19.85"N	5°10'46.75"W	August 2, 2012
2	Emlagh, Ireland	53°45'03.02"N	9°54'11.52"W	August 5, 2012
3	Holyhead, UK	53°19'09.85"N	4°39'41.92"W	August 3, 2012
4	Annascaul, Ireland	52°07'32.88"N	10°6'36.03"W	August 4, 2012
5	Wembury, UK	50°18'48.29"N	4°06'25.57"W	June 5, 2012
6	Landunvez, France	48°32'45.94"N	4°44'45.84"W	June 6, 2012
7	Batz-sur-Mer, France	47°16'18.18"N	2°28'36.68"W	June 4, 2012
8	Royan, France	45°37'41.85"N	1°03'45.31"W	June 3, 2012
9	Biarritz, France	43°29'03.84"N	1°33'47.74"W	June 22, 2012
10	Prellezo, Spain	43°23'37.63"N	4°26'25.25"W	June 21, 2012
11	La Caridad, Spain	43°33'55.24"N	6°49'41.41"W	July 7, 2012
12	Cabo Touriñan, Spain	43°02'34.65"N	9°17'24.30"W	July 8, 2012
13	Moledo do Minho, Portugal	41°50'23.83"N	8°52'26.37"W	July 5, 2012
14	Mindelo, Portugal	41°18'37.48"N	8°44'32.83"W	August 8, 2012
15	São Lourenço, Portugal	39°0'48.48"N	9°25'20.91"W	June 7, 2012
16	Alteirinhos, Portugal	37°31'09.33"N	8°47'20.91"W	June 5, 2012
17	Evaristo, Portugal	37°04'27.39"N	8°18'13.66"W	June 3, 2012

and 7.5% for the resolving gels for optimal protein resolution). To allow quantitative comparison between samples from different gels, we prepared a 'common sample' by mixing aliquots of several homogenates (totalling 1 mL). In each gel, two lanes were loaded with 15 µg of 'common sample', two 'standard' lanes were loaded with 80 ng of purified recombinant human HSP70B' protein (#ADI-SPP-762, Enzo Life Sciences, New York, NY, USA), one lane was loaded with 5 µL of prestained markers (#161-0374, BioRad) and the remaining eight lanes were loaded with the samples to be quantified (15 µg each). Gels were run at 100 V for 20 min followed by 120 V for 65 min in running buffer (1.4% glycine, 0.3% Tris-base, 0.1% SDS, pH 8.3).

Proteins were transferred to 0.45 µm polyvinylidene difluoride (PVDF) membranes (Millipore Immobilon #IPVH00010, Fisher Scientific, Boston, MA, USA) in transfer buffer (0.06% Tris-base, 0.03% glycine, 20% methanol), at 100 V for 35 min in a Mini Trans-Blot Electrophoretic Transfer Cell (#170-3930, BioRad). After transfer, membranes were treated with blocking buffer [0.1% nonfat dried milk in PBST solution (0.2% Tween, 8.5% NaCl, 0.2% monosodium phosphate, 1.1% disodium phosphate)] and then incubated for 10 min in a solution of both 1 : 1000 monoclonal anti-Hsp70 rat antibody (Thermo Scientific #MA3-006) and monoclonal 1 : 8000 anti- α -tubulin mouse antibody (Sigma-Aldrich #T5168) diluted in blocking buffer. Membranes were then washed three times with 15 mL of PBST solution, incubated with a 1 : 1000 solution of sheep-antimouse secondary antibody (#NA931, Sigma-Aldrich) diluted in blocking buffer and then washed three more times with PBST. Finally, membranes were exposed to an enhanced chemiluminescence solution (ECL, #RPN2106, GE Healthcare, Piscataway, NJ, USA) for 1 min and then exposed under darkroom conditions to ECL Hyperfilm (#28-9068-37, GE Healthcare) for 2–7 min to obtain various exposures that were in the linear range of detection. We processed digitized films

with IMAGEJ v1.4 (Abràmoff *et al.*, 2004). All samples were run at least three times.

Data processing and analysis

Quantitative western blotting across gels is sensitive to (i) variations in the efficiency of protein transfer and binding to the blotting membrane and to (ii) irregularities in the preparation and pipetting of samples, which can lead to inconsistent densitometry data (Taylor *et al.*, 2013). To control for variations in optical density and background noise typically found between gels, we quantified the optical density of the two 'common sample' bands relative to the average optical density of the bands in the two 'standard' lanes (those with a known amount of purified HSP70B') per gel. To account for loading uncertainties, we normalized the optical density of the Hsp70 by the optical density of a housekeeping protein band (α -tubulin) per sample. Then, normalized values were quantified relative to the optical density of the 'common sample' bands previously assessed for each gel.

We compared variation in Hsp70 levels with sampling location (random effect factor) and with exposure to solar radiation (fixed effect factor, sun vs. shade) using a two-factor analysis of variance (ANOVA). Because variances were not homogeneous (Cochran's test, $P < 0.05$), data were transformed by $\sqrt{\log(x+1)}$ prior to analysis. Hsp70 levels were regressed against the maximum temperature measured by robolimpets in the period that preceded tissue collection. Because the temporal dynamics of Hsp70 anabolism and catabolism in *P. vulgata* are still unknown, a conservative period of 2 weeks (which is roughly equal to the period of the neap-spring tidal cycle) was chosen.

The plot of Hsp70 as a function of temperature maxima prior to sample collection was used to find a temperature threshold above which Hsp70 levels increased significantly (i.e. was used to find the minimum temperature with

potential to trigger a stress response). Then, we used long-term robolimpet data (hourly temperatures collected between the summers of 2010 and 2014) to compute the average number of potentially stressful days per year (i.e. yearly mean number of days in which there were temperature registers higher than the temperature threshold) at sun-exposed and shaded microhabitats in the studied locations and regressed against populations densities in a potential limiting factor analysis (95th regression quantile estimation, Cade *et al.*, 1999; Cade & Noon, 2003). All data processing and analyses were performed in R 3.1.2 (R Core Team, 2014).

Results

Population densities of *Patella vulgata* were highest along the coasts of Great Britain and Ireland, decreasing southwards (see Fig 1b). With the exception of a single location in Ireland (#4, Fig 1b), average densities in shaded microhabitats were higher than average densities in sun-exposed rocky surfaces. We confirmed the absence of the species in the locations of Biarritz in SW France and Evaristo in SW Portugal (shores 9 and 17, respectively, Fig 1b). Northward from these locations, population densities were extremely low (just a few individuals per square metre, shores 8, 15 and 16, Fig 1b).

The Hsp70 antibody detected a single band in foot tissue of *P. vulgata*. Although we did not quantify its molecular mass, the migration pattern was similar to that of the recombinant human Hsp70 isoform. In this assay, and despite extensive modifications to the protocol, we were never able to reliably distinguish the constitutive from inducible forms of Hsp70. This has been reported in other studies, where, depending upon species, the two isoforms cannot always be distinguished (e.g., Schill *et al.*, 2002; Dong & Dong, 2008; Dong *et al.*, 2008; Dong & Williams, 2011; Dalvi *et al.*, 2012). However, because both forms play a role in organismal thermal tolerance, the quantification of total Hsp70 levels summarizes the organism ability to cope with thermal stress, and thus has been considered more informative from an ecological point of view (Sorte & Hofmann, 2005; Sagarin & Somero, 2006). Consequently, we report here the combined quantity of both the constitutive and inducible forms of Hsp70.

The two-factor analysis of variance showed both a significant effect of location ($F_{14, 210} = 9.89$, $P < 0.01$) and exposure to the sun ($F_{1, 14} = 96.93$, $P < 0.01$) in the endogenous levels of Hsp70, but no significant interaction between location and solar exposure (see Table 2 for details). In fact, within each location, the average level of Hsp70 among individuals collected from sun-exposed microhabitats was consistently higher than the average level of those collected in the shade (Fig. 1a). Hsp70 levels from animals collected in sun-exposed microhabitats on an average were 1.9 times higher than

Table 2 Two-factor ANOVA to measure the effect of exposure to solar radiation (sun vs. shade) and sampling location on the endogenous levels of Hsp on *Patella vulgata*

Source of variation	SS	DF	MS	F	P	Against
Location	1.041	14	0.074	9.888	<0.01	Error
Sun exposure	1.08	1	1.08	96.927	<0.01	Location × sun exposure
Location × sun exposure	0.156	14	0.011	1.481	0.12	Error
Error	1.58	210	0.008			
Total	3.857	239				

the levels found in animals collected in shaded microhabitats. Hsp70 showed no apparent correlation with latitude (Pearson's correlations: $r = -0.33$, $t = -1.25$, $df = 13$, *n.s.* for sun-exposed individuals and $r = -0.34$, $t = -1.31$, $df = 13$, *n.s.* for shade-exposed individuals, see also Fig. 1a). Hsp70 levels were particularly high at the locations immediately north of the distributional gap in the Bay of Biscay (#8, Fig. 1a) and north of the distributional limit in southern Portugal (#16, Fig. 1a). In effect, at these locations, individuals from sun-exposed microhabitats had, on an average, the highest levels of Hsp70 in the whole range, and even animals from shaded microhabitats at their distribution limit in Portugal displayed Hsp70 levels comparable to sun-exposed habitats elsewhere.

Hsp70 levels were significantly correlated with maximum body temperatures measured by robolimpets during the 2 weeks that preceded tissue collection (Pearson's correlation $r = 0.70$, $t = 5.15$, $df = 28$, $P < 0.05$). The scatterplot in Fig. 2 shows two main groups. In general, limpets from shaded microhabitats (light grey dots) experienced relatively low temperatures and had low levels of Hsp70, irrespective of their geographical origin. Conversely, animals from sun-exposed microhabitats (dark grey dots) experienced high temperatures prior to collection and had elevated levels of Hsp70. Interestingly, the only two cases of sun-exposed individuals with low levels of Hsp70 (dark grey dots #4 and #10) match the overall pattern because they also experienced relatively cold temperatures prior to collection. It was also remarkable that even locations thousands of km apart (such as #1 and #13) grouped together in both extremes of the graph, reinforcing the idea that geographical location had little contribution for the overall pattern shown in Fig. 2. Locations close to the geographical limits (#8 and #16) matched the overall pattern, but had Hsp70 levels approximately 1.5× to 2× higher than what would be predicted given the temperatures experienced in the 2 weeks before

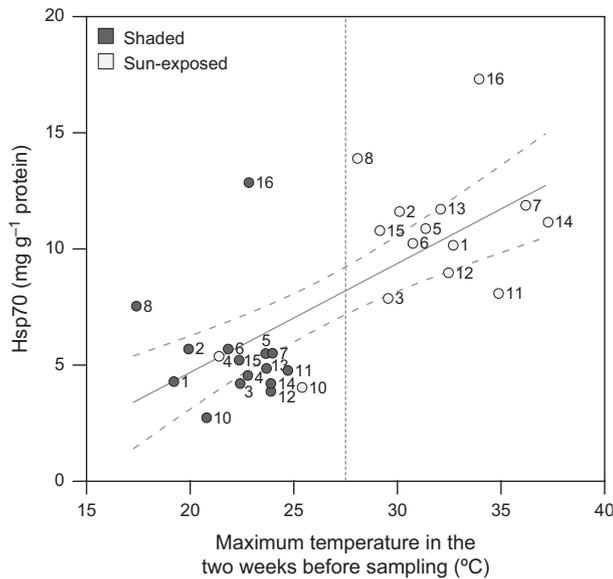


Fig. 2 Correlation between Hsp70 and temperatures experienced prior to sample collection. Each dot represents either a shaded microhabitat (light grey) or sun-exposed microhabitat (dark grey). Reported Hsp70 is the average level among 8 individuals collected at each microhabitat type within each location. Curved dashed lines enclose the 95% confidence interval. Reported temperature is the maximum temperature measured by robolimpets in 2 weeks that preceded tissue collection for Hsp70 quantification. Vertical dotted line shows the temperature threshold of 27.5 °C used in Fig. 3. See Fig. 1 (c) for a map with sampled locations.

collection. Finally, the Hsp70 vs. temperature plot suggested that ~ 27.5 °C could be used as an estimate for the minimum stressful temperature, above which a marked increase in Hsp70 levels might be expected.

The 'wedge-shaped' distribution of *P. vulgata*'s densities plotted against number of potentially stressful days (i.e. the average yearly number of days with maximum temperature above 27.5 °C, see Fig. 3) was consistent with the hypothesis that high temperatures act as a limiting factor for *P. vulgata* (Southward *et al.*, 1995; Cade *et al.*, 1999). The potential limiting factor analysis suggested that the 95th quantile of the densities in the field were significantly and inversely correlated with the average yearly number of days with maximum temperature above 27.5 °C, and that each additional hot day per year reduced the maximum potential density of *P. vulgata* by -2.14 ± 0.34 SE individuals m^{-2} ($P < 0.05$ after 1000 bootstraps).

Discussion

Our study was designed to examine the importance of thermal refugia in the geographical distribution of an

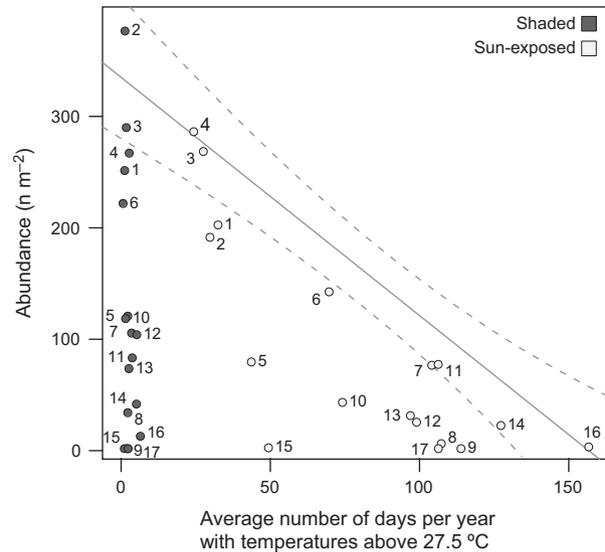


Fig. 3 Temperature as a potential limiting factor for *Patella vulgata*. The solid line shows the 95th regression quantile estimate for the abundance of *P. vulgata* as a function of the yearly number of days exceeding 27.5 °C. Dashed lines enclose the 95% confidence interval. The temperature threshold of 27.5 °C was obtained from Fig. 2 (see Material and methods). Number of days based on 1 h-resolution robolimpet data collected between the summers of 2010 and 2014. Each data point represents either a shaded microhabitat (light grey) or sun-exposed microhabitat (dark grey). Reported abundance is the mean density of *P. vulgata* quantified in 15 quadrats of 30 × 30 cm at each microhabitat type within each location. See Fig. 1 (c) for a map with sampled locations.

intertidal species. We asked whether differences between sun-exposed and shaded microhabitats were responsible for differences in physiological stress and ecological performance and examined the availability of refugia near equatorial range limits. Using the HSP response and population densities as proxies, we show that limiting effects of temperature, rather than being related to latitude, seem to be tightly associated with microtopography.

Microhabitat-associated variability in thermal stress

Our study encompassed the majority of the geographical range of *Patella vulgata*. Despite the wide latitudinal breadth, there was not a single location where animals inhabiting sun-exposed rock surfaces did not have, on an average, higher Hsp70 levels than animals collected from the corresponding shaded microhabitats (Fig 1a). This is remarkable, especially considering the site to site variability introduced by the geographical extent of the study (with locations featuring different geomorphology, exposure to wave action, food availability, or

community composition among others). Taking into account the combined metabolic cost of replacing heat damaged proteins (Hofmann & Somero, 1995) and the heat-shock response itself (including the expenditure of ATP in heat-shock gene transcription, synthesis of Hsp and maintenance of their chaperoning functions, Somero, 2002), the present data suggests that thermal differences between sun-exposed and shaded microhabitats (Figs 2 and 3) are indeed associated with differences in physiological performance, and thus have the potential to significantly affect processes such as survival, growth or reproduction.

Biogeographical consequences of recurrent thermal stress

Our data show that the contrasting temperatures between sun-exposed and shaded microhabitats previously reported by Seabra *et al.* (2011) for the Iberian Peninsula are common over much wider spatial scales (at least over the majority of the European Atlantic coast), and over larger temporal scales (at least four years). Temperatures at sun-exposed microsites routinely exceed 27.5 °C even at high latitudes; for example, sun-exposed microsites in the British Isles had, on an average, approximately 30 such days per year. In contrast in shaded microhabitats, temperatures hardly ever exceeded 27.5 °C, even at the southernmost locations, in southern Portugal (Fig. 3).

Our study suggests that the frequency of occurrence of high temperatures imposes a limit on the maximum population densities supported at any given place. We hypothesize that: (i) physiological stress increases when animals are exposed to high temperatures and that (ii) because higher temperatures are recurring in sun-exposed microhabitats, ecological outcomes of heat stress (such as diminished population densities) are also more severe in sun-exposed microhabitats. Thus, the limiting effects of temperature, rather than being related to latitude, seem to be tightly associated with microsite variability, reinforcing the notion that the physiological responses of intertidal organisms (and subsequent ecological consequences) are driven by the environmental conditions observed in their immediate vicinity (Helmuth *et al.*, 2010). If these hypotheses are correct, then population densities of septentrional (cold-water) species at a given location are largely controlled by the relative abundance of hot (stressful) and cold (refugia) microhabitats. Locations with appropriate geomorphology (e.g. north-facing cliffs) or with adequate topographical complexity (e.g. large boulders with high thermal inertia) should harbour larger populations because they allow its inhabiting organisms to withstand thermally stressful periods during diurnal low tide (Gedan *et al.*, 2010). This may explain why the

'abundant-centre' distribution, with highest population densities at the centre of the distribution range and declining towards the range edges, has rarely been observed in intertidal species (Sagarin & Gaines, 2002a, b; Pironon *et al.*, 2015), as the fraction of the local habitat that can be used as thermal refugia is not necessarily correlated with latitude.

In *P. vulgata*, thermal stress in the vicinity of the hot range limits (both immediately north of the distribution gap in the Bay of Biscay and north of the absolute distribution limit in SW Iberia) is so high that although we found the same general pattern between microsites (i.e. limpets exposed to solar radiation are, on an average, more stressed), even animals from shaded microsites are unable to avoid severe thermal stress. Thus, at the hot range edges, the magnitude of the amelioration provided by the shade is insufficient to provide thermal refugia, which could be the very reason why the geographical limits are there. The scarcity of thermal refugia and the extremely low population densities means that larval supply and thus recruitment are most likely to be compromised, which is probably made worse by a shortage of males at those latitudes (Borges *et al.*, 2015). Figure 2 suggests that the reason for the increased thermal stress at these marginal populations is not exclusively related to maximum temperatures occurring immediately prior to collection (note how both shaded and sun-exposed data points #8 and #16 are higher than expected considering only maximum temperatures). It is plausible that a second environmental stressor (or combination of stressors), such as oxygen deprivation, desiccation, salinity extremes, or pollutant contamination (which are known to increase Hsp70 production, Feder & Hofmann, 1999) are imposing additional layers of stress on these marginal populations. We speculate, however, that the cause for the increased stress is the elevated seawater temperature characteristic of these locations during summer. In fact, in that season, both the coastal waters off southern Portugal and in the southern Bay of Biscay are warmer than the surrounding regions (Valencia *et al.*, 2004; Lima *et al.*, 2007). This explanation corroborates previous studies indicating that the equatorial range edge for this species is set by summer conditions (Bowman & Lewis, 1977) and is congruent with the fact that both sun-exposed and shaded microhabitats exhibited abnormally high stress levels (as both microhabitats are equally submerged during high tide). Furthermore, the proposed mechanism is supported by recent experimental data showing that water temperatures at these locations elicit remarkably high levels of thermal stress, effectively acting as a threshold beyond which *P. vulgata* cannot exist (Seabra *et al.*, submitted). The same study suggests that individuals from his species living

in regions such as NW Iberia and NW France, routinely exposed to cold upwelled water during summer, have opportunity to recover during high tide.

Evolutionary implications

If shaded microhabitats can be found in almost every location why have organisms not evolved to either selectively settle in or to subsequently move to shaded microhabitats? Although that might be the case for some species (e.g. the barnacle *Perforatus perforatus*, the sponge *Hymeniacidon sanguinea* or the anemone *Actinothoe sphyrodeta* which can only be found in crevices or shady rocky overhangs), it does not appear to happen with *P. vulgata*. These limpets have greater population densities in shaded microsites (Fig. 1b) which means that intrageneric and intraspecific competition for food and/or space is much higher in those microhabitats (Boaventura *et al.*, 2002, 2003; Espinosa *et al.*, 2006). In addition, sun-exposed surfaces have more light availability, and thus, within reasonable limits, have the potential to sustain higher primary productivity, which is beneficial for herbivores such as limpets (Einav *et al.*, 1995; Harley, 2002). This hypothesis is supported by recent data suggesting that grazing pressure from limpets is much greater on south- than on north-facing substrata (Firth *et al.*, 2015). This may, however, not be valid in extreme situations because biofilm growth is inhibited by extreme insolation during summer (Thompson *et al.*, 2004). Thus, even though living in sun-exposed microhabitats means having to cope with higher levels of thermal stress, other factors, such as reduced competition, increased food availability or even shelter offered by canopy forming algae (Moore *et al.*, 2007) may play important ameliorating roles, reducing the selective pressure from choosing shaded microhabitats.

Importance for climate change

The variability in thermal stress arising from local topography means that, throughout their entire distributional range, most sessile intertidal species are exposed to a mosaic of highly stressful areas interspersed with thermal refugia. This heterogeneity is likely to have profound effects on the way local populations (and therefore species as a whole) respond to either long-term increases in temperature (Lima & Wethey, 2012) or to increases in the frequency of extreme events (Wethey *et al.*, 2011). The resilience or vulnerability of local populations to changes in climate is probably highly dependent on the abundance of thermal refugia, which may act as a thermal buffer. These refugia provide conditions that are at or below

the point of collapse of the thermal performance curve (Woodin *et al.*, 2013), overriding the long-term warming trend and allowing populations of northern species to persist, thus maintaining the overall extent of the range despite patchy extirpations in thermally stressful microhabitats. The availability of thermal refugia may even allow the ranges of northern species to be extended equatorward to areas otherwise uninhabitable. Species with meridional affinity (warm-water species), on the other hand, benefit from hot microhabitats and thus the expansion of their ranges northwards can be assisted by the greater availability of appropriate microhabitats with global warming. In fact, contrasting patterns in the average direction of change between cold- and warm-water species (including counterintuitive equatorward range expansions of septentrional species) have already been described for a wide range of sessile intertidal organisms (e.g. Lima *et al.*, 2007; Hilbish *et al.*, 2010). In the specific case of *P. vulgata*, during the last glaciation the species occurred much further south, then retreated polewards during the transition to the warmer interglacial climate, and finally re-expanded during the glacial-like conditions of Younger Dryas (see Ortea, 1986; Southward *et al.*, 1995). Small-scale thermal refugia was most probably fundamental for the continued recruitment of *P. vulgata* towards its range edge.

Since the effects of topography on site conditions have the potential to locally override the effects of global warming (Holtmeier & Broll, 2005), it is necessary to understand the link between the environmental mosaic and macroecological processes to correctly forecast the consequences of climatic change, a task which may be more complex than could be anticipated (Marshall *et al.*, 2013). A comprehensive understanding of the abundance or scarcity of refugia (including the consideration of multiple stressors or multiple aspects of a single stressor, Seabra *et al.*, 2015) is thus crucial to predict where and when the effects of climate change will occur.

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