Usage guidelines

A biologically relevant rapid quantification of physical and biological stress profiles on rocky shores

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ABSTRACT

Different combinations and intensities of physical (e.g. thermal) and biological (e.g. competition or predation) stress operate on organisms in different locations. Variation in these stresses can occur over small to medium spatial scales (cm to 10s of metres) in heterogeneous environments such as rocky shores, due to differences in sun and wave exposure, shore topography and/or recruitment. In this study we demonstrate how simple measurements can be taken that represent physical and biological stresses (stress profiles) in a given location. Using a bootstrapped principal component analysis, we identified significantly different stress profiles at four sites separated by only 10s to 100s of metres on the Shek O peninsula in Hong Kong. We then measured response to thermal stress, as determined by detachment temperature, in the limpet *Cellana grata* (which is known to be a sensitive indicator species to thermal stress) from each location. Significant differences in stress profile between locations were also seen in thermal stress tolerance of limpets from those locations. At locations where the major stresses are likely to be more physical or less biological in nature (e.g. southerly facing aspect or lower density of grazers), the mean detachment temperature was higher, whereas detachment temperature was lower at sites with more biological or less physical stress. This method is, therefore, able to determine biologically meaningful differences in stress profiles over small to medium spatial scales, and demonstrates that localised adaptation (i.e. post planktonic settlement) or acclimation of species may occur in response to these different stress profiles. The technique can be adapted to different environments and smaller or larger spatial scales as long as the stress experienced by the study species is relevant to these scales.

1. Introduction

On rocky shores few organisms live at environmentally optimal conditions, and are often forced towards the edges of their fundamental niche by biological factors such as predation and competition (Lubchenco, 1980; Somero, 2010; Underwood and Denley, 1984). Examples of these realised niches are common in the rocky shore literature, where shore height (and hence relative immersion:emersion time) provides a simple and localised environmental gradient (Colman, 1933; Harley and Helmuth, 2003). For example, transplants of algae to lower shore levels in the absence of grazers and other competitive species typically result in much higher growth rates of algae than at the shore height they normally occur (Hawkins and Hartnoll, 1985; Norton, 1985), indicating that these species usually occur outside their optimal environmental conditions.

Typically, for hard substrate marine communities there is considerable competition for space (reviewed by Paine, 1984), and as a result, many organisms will be forced towards the edges of their fundamental

niches (Braunisch et al., 2008; Costantini et al., 2009). To contend with this, organisms on rocky shores must either tolerate less favourable environmental conditions such as longer periods of desiccation or thermal stress, or have the ability to be able to cope with higher levels of competition, grazing or predation (van Straalen and Roelofs, 2012).

In the rocky intertidal zone, different physical locations (shores or sites within shores) present very different sets of biological and physical challenges for a given species, and these differences can change over a range of spatial scales (see examples in Harley and Helmuth, 2003). For example, at headlands and peninsulas both wave exposure and direct sunlight (and hence temperature and desiccation levels) can vary dramatically over 10s of metres (for example, between north and south facing shores). The impact of predators, competitors and facilitators can also vary over these spatial scales (Menge et al., 1994), often as a result of differential physical factors (Benedetti-Cecchi, 2001). Such distances are normally greater than a typical organism will displace in its adult lifetime, especially when the rocky substratum is not continuous (i.e. separated by sand patches).

The classic theoretical models of Menge and Sutherland (1976, 1987) propose that the roles of physical and biological factors such as disturbance, competition and predation, differ with environmental

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stress. Since physical stress differs at a local scale of 10s of metres (due to aspect or wave exposure), this infers that biological stresses should also vary at these scales (Benedetti-Cecchi, 2001; Harley and Helmuth, 2003), as do patterns of community structure (e.g. Hutchinson and Williams, 2001).

Adaptations to these combinations of stress (herein 'stress profiles') are likely to occur to maximise survival and reproduction (and hence fitness). Such adaptations could occur through natural selection, however, given that most rocky shore organisms have a planktonic dispersal phase (Pechenik, 1999), localised adaptations (over distances measured in 10s or 100s of metres) are likely to be acclimation responses rather than evolutionary responses (Allcock et al., 1997; Hoskin, 1997; Somero, 2010; but see Krueger-Hadfield et al., 2013).

In this study, we characterise small sections of shore separated by short distances (10s–100s of metres) in relation to a number of physical and biological parameters, to determine the combinations of physical and biological factors that influence the stress profile of organisms. Using a bootstrapped PCA approach (modified from Catlin-Groves et al., 2009; Stafford et al., 2012), we test for significant differences in types of stress acting on limpets (Cellana grata) on four sections of shores (each 10 m long, but all separated by <500 m) on the Shek O peninsula on Hong Kong Island. We collected limpets from these sections and tested their response to thermal stress by investigating detachment temperatures. We hypothesise that, where stress profiles differ between sections of shore, those sections where physical parameters are more important (i.e. a lower density of grazers, or a southerly aspect), will have limpets with higher detachment temperatures (i.e. animals are more resistant to temperature extremes). Equally, where stress profiles are more associated with biological stress and less with physical stresses (e.g. higher grazer density meaning more competition, northerly aspect), limpets will have lower detachment temperatures. Support for these hypotheses would indicate that the calculation of

stress profiles is biologically meaningful and a useful tool to investigate differences in stress profiles over small spatial scales.

2. Methods

Four sites (continuous sections of rocky shore of 10 m in length) were selected on the Shek O peninsula on Hong Kong Island (Fig. 1) in late May 2013. Sites were selected based on initial inspection of community structure, and knowledge of the direction they faced as well as predominant wave conditions to ensure a variety of potential physical and biological stresses were captured. A 10-m line transect was placed at the height on the section of shore with the highest density of C. grata (this shore height differed between sites, but was recorded by measuring the vertical distance from the sea using a cross staff, see Baker and Crothers, 1987 for details). The GPS position (accuracy ±3 m) was noted at each site, and a number of parameters measured. Grazer density (for C. grata and combined for all other grazers) was obtained from five haphazardly placed 50 × 50 cm double strung quadrats along each 10 m transect. Crevice and rockpool percentage cover and barnacle and mussel percentage cover were also recorded from these five quadrats using the point intercept technique (as described in Bohnsack, 1979). Five recordings of shore slope were taken, using a clinometer placed in the centre of each quadrat (one recording per quadrat). This sampling covered 50% of the area being considered, which has been shown to be sufficient to capture details of even the most patchily distributed organisms or shore characteristics (Stafford, 2002). Photographs of the site, in direct sunlight with a white sheet of paper in the field of view were also taken to determine rock colour (see below).

Simple computer-based analysis followed the field-based data collection. Sites were scored on an ordinal scale for exposure to sun (north facing = 1, east = 2, west = 3, south = 4) and wave exposure (west = 1, north = 2, south = 3, east = 4). Wave exposure was
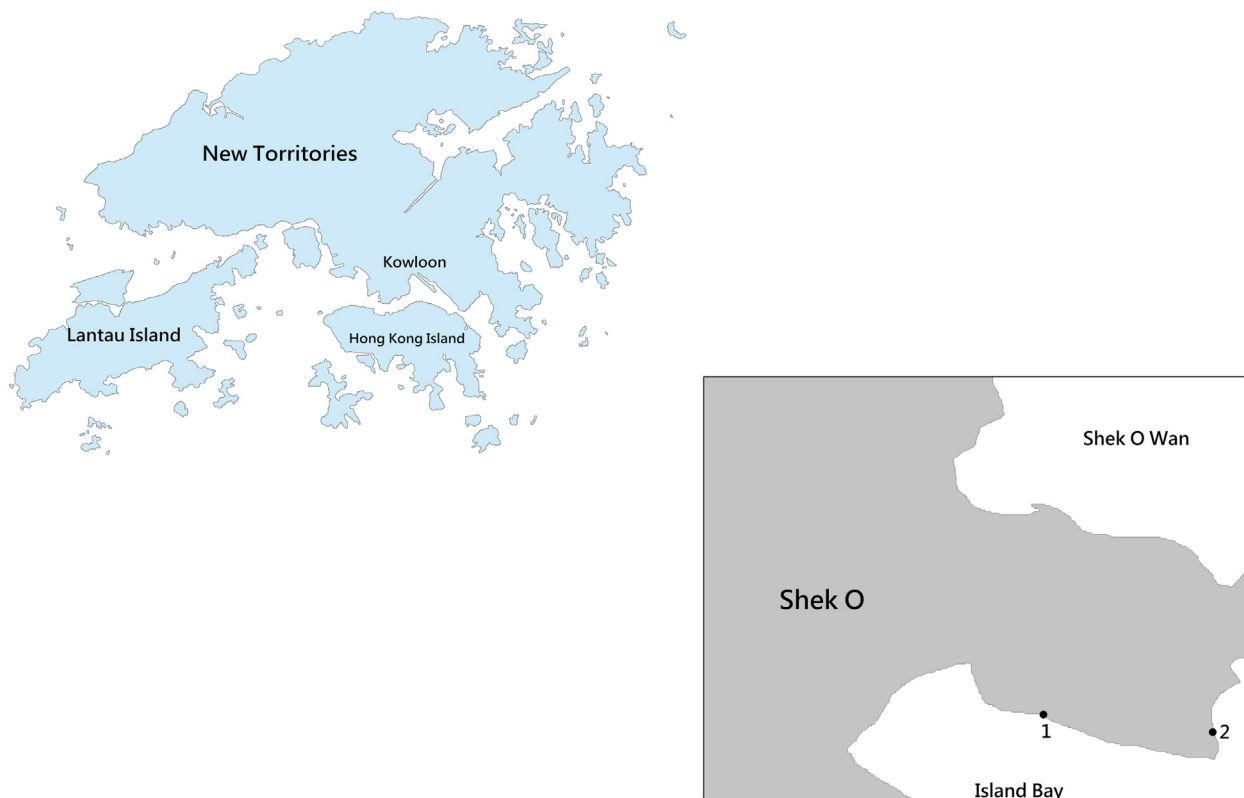

Fig. 1. Locations of the four sites on the Shek O peninsula on Hong Kong Island.

based on predominant swell direction for Shek O, obtained from seasonal data available from MetCentral Ltd and from analysis of fetch from the sites (i.e. shelter from headlands or near shore islands reduced the exposure of north facing beaches). Photographs of the site were analysed to determine the rock colour using the GIMP image analysis package (www.gimp.org), by converting to greyscale, transforming the image so the white paper had a pixel colour value of 255, then calculating the mean pixel colour value of 10 rock sample points (average of 4×4 pixels) to determine the darkness of the rock (converted so that darker values have higher values by subtracting the mean value obtained from 255).

Raw data for each site were transformed into a percentage weighting for each measured parameter. This presented data from different variables on a uniform scale, but also was fundamental to the assumption that all sites are stressed, just by different factors or magnitudes of those factors. For example, if a site faced south, yet had few other grazers, the importance of the direction in terms of thermal stress would be emphasised and the contribution of biological stress would be reduced. To achieve this percentage weighting; 1) all measured variables at a given site were standardised by dividing by the mean value for that variable across all sites. 2) The percentage value of each factor at each site was then calculated by dividing the standardised values, obtained in step 1, of each factor at each site by the sum of all factors for each site and then multiplying by 100 (see Supplementary material for details of calculation). This way, the standardised percentage weighting of each factor for each site was determined (Table 1).

Data were analysed using a bootstrapped principal component analysis (see Stafford et al., 2012 for full details). Briefly, sites were classified by 100 data points, where x of those points were equal to the percentage of the considered component of the physical and biological factors at each site (i.e. the first factor is density of C. grata, the second the density of other grazers and so on, following the order of parameters given in Table 1).

Samples were then taken (with replacement) of these 100 data points for each site (100 points were sampled, but because this was with replacement, many would be sampled more than once, and many not at all, following recommended bootstrapping procedures in Efron, 1979). From this sample, the first three principal components were calculated. This process of determining the first three principal components was conducted 10,000 times (with appropriate transformations to allow for the arbitrary nature of principal component axes, and 'reflection' of the axes — full details of this are given in Stafford et al., 2012). The samples that were the furthest 5% from the mean PC value for the site were then excluded to provide 95% confidence spheres on a three dimensional plot of the first three principal components. An overlap of these spheres indicates no significant difference between the stress regimes of the corresponding sites (see Stafford et al., 2013,

for discussion of overlapping of bootstrapped-derived 95% confidence limits for determining significance). The technique was developed to determine differences between limited cases, and hence the variable to case ratio can easily exceed 1 (Stafford et al., 2012). The limitation of the technique is that the variables need to be appropriately compressed so that the first three principal components explain >90% of the variability in the data, otherwise the positions of the spheres may not be reliable enough to determine significant differences (Stafford et al., 2012). The code for running the bootstrapped PCA is provided at http://rickstafford.com/software/software.html. To explore the critical differentiating factors between the sites, a biplot from non-bootstrapped PCA was produced. However, this plot should be treated with some caution, as the case to variable ratio for PCA is exceeded (Tabachnick and Fidell, 1989).

Two days after taking shore measurements, C. grata were collected from each site (all C. grata from all sites collected within 90 min on the same day). Where possible 10 individuals in the size range 20–28 mm were collected. Where this was not possible, due to restricted numbers of limpets in this size range, as many as could be obtained were collected (minimum n = 7). Limpets were stored at ambient temperature for no more than 90 min before laboratory experiments. Initially all limpets were placed in plastic pots (diameter 40 mm) in air and then refreshed under a filtered and aerated seawater spray for 5 min on return to the laboratory. All limpets attached firmly to the bottom of the plastic pots. The pots were then drained of water, sealed, and placed upside down in a water bath, initially at 30 °C, and maintained at 30 °C for 2 min, before the temperature was raised by 1 °C every 2 min. A measure of thermal tolerance was obtained by observing when limpets lost adhesion and fell from the top of the pots (the detachment temperature, as described by Wolcott, 1973, after which he considered limpets 'ecologically dead'). Detachment temperatures were analysed by multiple regression, with size of limpet as a linear continuous variable, and site from which the limpet was collected a factorial variable in the analysis. Ideally we would have collected limpets in a narrower size range, then excluded 'size' as a variable in the model, and just tested for differences in mean detachment temperature between sites. However, due to the restricted number of limpets at each site, and differing, but overlapping sizes between sites, we included size as a variable in case this directly influenced detachment temperature.

3. Results

The bootstrapped PCA process indicated that two sites were statistically similar in terms of their physical and biological characteristics (i.e. their spheres overlapped), and two sites were significantly different from each other and from the two similar sites (i.e. spheres did not overlap; Fig. 2). The first three principal components explained 97.7% of the

Table 1
Physical and biological measurements of each site. Raw data collected in situ or from examination of site aspect or photographs and the percentage contribution of this parameter to the overall stress profile of the site (see the Methods section for details). The final column indicates the rationale for choosing this measurement.

| Variable	Raw data Site 1	Raw data Site 2	Raw data Site 3	Raw data Site 4	Percentage contribution Site 1	Percentage contribution Site 2	Percentage contribution Site 3	Percentage contribution Site 4	Rationale
Limpet (mean number per quadrat)	13.6	38.6	23.2	4.6	8	15	10	2	Intraspecific competition
Other grazers (mean number per quadrat)	4.4	3.0	23.6	3.8	6	3	24	4	Interspecific competition
Barnacle/mussel (mean percentage cover)	1.0	45.6	0.0	9.8	1	28	0	7	Facilitation and provision of biotic microhabitats
Crevice/rockpool (mean percentage cover)	6.2	5.2	18.4	15.4	9	5	20	17	Provision of abiotic microhabitats
Angle (degrees from horizontal)	28.4	31.8	63.4	24.2	10	7	16	6	Related to potential shade and water run off
Shore height (m + C.D.)	2.0	1.85	1.2	2.2	17	10	7	15	Time of emersion
Colour (mean pixel value)	135	99	111	144	17	8	10	14	Heat absorption
Insolation index (1–4)	4.0	3	1.5	4.0	20	10	6	16	Exposure to direct sunlight and associated temperature
Exposure index (1–4)	2.0	3.5	1.6	4.0	12	14	7	19	Exposure to wave action and possible modification of effective shore height
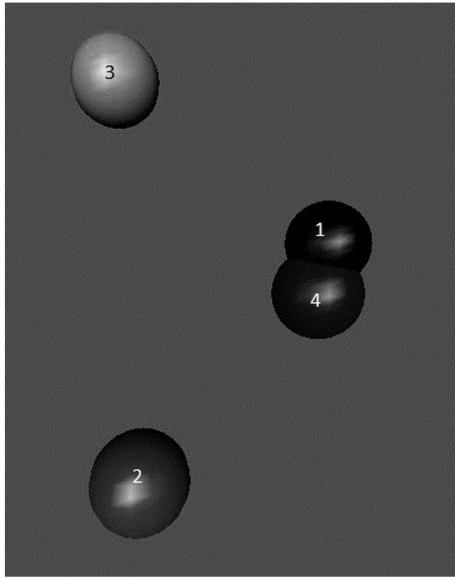
Fig. 2. Bootstrapped PCA (with 95% confidence spheres) of the measured physical and biological variables at the four sites (1–4). Overlap indicates that no significant difference occurs between the sites.

Table 2

Results of multiple regression analysis for detachment temperature of limpets against size of limpet and the site it was collected from (a) for all four sites. Results for different sites demonstrate significant differences compared to site 1. (b) For sites 1 and 4 combined (as these sites were shown not to differ significantly in stress profile). Results for different sites demonstrate significant differences compared to the combined sites 1 and 4.

	Estimate	S.E.	t	p
a)				
(Intercept)	30.2021	6.4871	4.656	<0.0001
Size	0.3560	0.2888	1.232	0.2297
Site 2	−3.8428	1.3549	−2.836	0.0091
Site 3	−4.2801	1.6687	−2.565	0.0170
Site 4	−2.1689	1.6335	−1.328	0.1968
b)				
(Intercept)	32.2021	6.4053	5.027	<0.0001
Size	0.2285	0.2765	0.826	0.4164
Site 2	−2.9862	1.2095	−2.469	0.0207
Site 3	−3.2885	1.5148	−2.171	0.0396

variability in the data. An examination of the biplot showed that sites 1 and 4 were discriminated from the other sites by increased physical stress, especially insolation, and also showing lighter rock colouration (Fig. 3). The importance of biological stress was reduced in these sites whereas site 2 had an increased number of limpets and site 3 had increased numbers of other grazers (Fig. 3).

During the detachment temperature experiment, ~10% of limpets crawled down the side of the pots, and as such, detachment temperature could not be obtained for these individuals. Two limpets did not detach from the pots due to adhesion with mucus, but were found to be dead after termination of the experiment at 60 °C, and these limpets were excluded from the analysis. All remaining limpets detached before 40 °C. Overall, 8 limpets provided data from site 1, 10 from site 2, 5 from site 3 and 6 from site 4. The regression for detachment temperature including the factor 'site' and the continuous variable of limpet size was significant with an adjusted $r^2 = 0.205$ (Table 2a). There was no
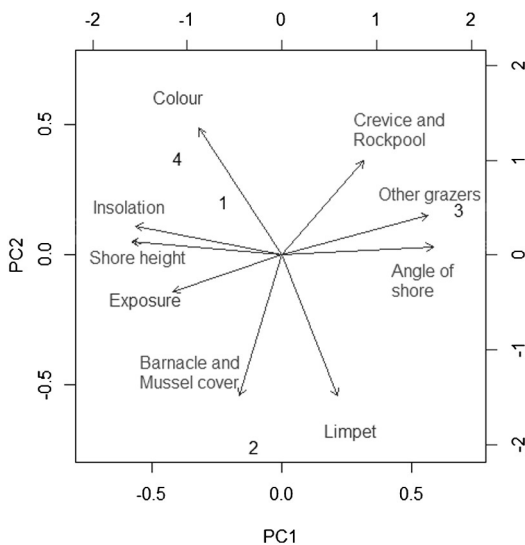
effect of limpet size on detachment temperature, but there was a difference between the sites the limpets were collected from and their detachment temperatures (Table 2; Fig. 4). Sites 1 and 4 did not differ significantly from each other, but sites 2 and 3 were significantly different from site 1 (Table 2a; Fig. 4). In a separate analysis, sites 1 and 4 were combined (giving 14 limpet detachment temperatures), because these sites were not significantly different according to the bootstrapped principal component analysis. Again, the regression was significant, but explained slightly less variability ($r^2 = 0.181$), in this case, detachment temperatures of limpets from sites 2 and 3 were significantly different from the combined site, but not from each other (Table 2b; Fig. 4).

4. Discussion

The results support the hypotheses presented in the introduction; namely that shores with relatively higher levels of physical stress such as having southerly aspects would have higher limpet detachment temperatures than sites with relatively higher levels of biological stress. Sites 1 and 4 had lower proportions of intra- and inter-specific competition, as measured by grazer density, than sites 2 and 3, indicating lower relative levels of biological stresses such as competition. These sites were also likely to have higher levels of physical stress, being darker in colour and higher on the shore than sites 2 and 3. While the proportion of insolation was lower at site 1 than at sites 2 and 4, both sites 1 and 4 were south facing, whereas site 2 was north facing and site 3 west facing.

Using a principal component analysis technique to determine differences between shores does demonstrate the inter-relatedness of some variables. For example, sites 1 and 4 showed higher levels of insolation,
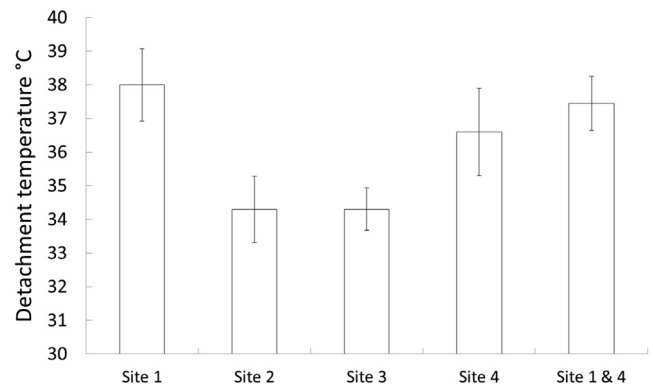

Fig. 3. Biplot of non-bootstrapped PCA based on percentage data in Table 1. Arrows indicate key discriminatory factors between sites (e.g. site 3 is discriminated from other sites by a greater number of other grazers and a steeper angle of shore). Note, variable to case ratio is exceeded for PCA (see the Methods section).

Fig. 4. Mean (±S.E. n = 8, 10, 5, 6 for sites 1–4, respectively) detachment temperature of limpets from each site. The final bar indicates the combination of sites 1 and 4 (n = 14) as these shores did not demonstrate significantly different stress profiles.

yet by examining the biplot, this was closely related to shore height, exposure and colour of the rock. In some cases, these relationships between factors might be slightly counterintuitive. For example, wave exposure might mean reduced desiccation or temperature stress, if the rock surface were wet by waves during emersion. However, *C. grata* suffers high levels of mortality due to acute physical stress, often occurring over one or a few consecutive tidal cycles in mid-summer (Ngan, 2006; Williams and Morritt, 1995). At these times, insolation and temperature would be very high, and normally related to high pressures and calm seas.

Colour was measured as the whiteness of the rock, and as such, it may be related to lack of biofilm. Because of this, the variable space largely divides into physical and biological factors with the top left corner of the biplot being dominated by physical factors. Differences between sites 2 and 3 largely occur because of differences in water retaining features and complexity (rockpools and crevices vs mussel beds and barnacles) and the type of competition occurring (intra- vs. inter-specific). It is unclear if these differences could be important in determining stress acting on the shore, and further details of adaptation to biological stress may need to be measured to determine this — in this study shores with higher physical stress do show differences in responses of their limpets to detachment temperature.

This study demonstrates that the stress profile of a limited section of rocky shore can be described by a small number of simple field parameters augmented by follow-up image analysis of photographs. Using open source statistical techniques, it is possible to determine significantly different stress profiles on spatially proximate sections of shores. These calculated stress profiles demonstrate real biological relevance, because the heat tolerance of limpets from these different stress profiles also differed significantly and in a predictable manner based on the measured stress profiles.

Predicting different stress profiles at small spatial scales is important. Traditional broad scale (10s–100s of kilometres) climate envelope and 'niche' models, which largely concentrate on air temperatures and rainfall (e.g. Peterson, 2001) are proving ineffective at mapping fine scale species distributions, leading to the development of smaller microclimate models (Gillingham et al., 2012a, b). However, biological factors such as competition, predation, parasitism and facilitation are known to be important in determining the ecological niche of most organisms (Leibold, 1995). Despite the recognised importance of biological factors, determining the strength of such interactions is difficult and time consuming without intensive manipulative experiments or modern modelling approaches (reviewed by Underwood, 2000; Wooton and Emmerson, 2005).

The technique presented here provides a rapid, largely in-situ method to determine stress regimes, indicative of the biological and physical factors acting on organisms. Localised acclimation by limpets also appears to result from these different stress regimes (possibly as a result in changes in heat shock proteins Halpin et al., 2002; Harley et al., 2009). Understanding that in limpets different types of acclimation may occur on different sections of shores where the factors structuring communities and acting on individuals differ significantly (for example, a greater proportion of competition vs. a greater proportion of physical stress through direct insolation at different sections of the shore), and may not only be related to large scale variables such as latitude, is important in understanding species responses to disturbances such as climate change (see discussion in Helmuth, 2009).

Many studies are recognising the need for incorporation of biological and physiological processes along with environmental variables. The notion of the 'physiological niche' has become popular, with many authors realising it may be an interplay of physical and biological processes that determine species distributions, growth rates and ultimately evolutionary fitness (e.g. Helaouët and Beaugrand, 2009; Helmuth, 2009; Kearney and Porter, 2009; Sarà et al., 2011). However, the approach detailed in this paper of classifying micro- or meso-habitats as

significantly different in the makeup of physical and biological factors can be used to rapidly determine differences in realised niches of organisms in the field. Significant differences detected between sites can also validate the use of 'natural experiments', where different sites are compared, rather than true manipulations being carried out (Diamond, 1983; Sagarin and Pauchard, 2010). Using this field technique along with measures to predict and measure physiological limits of study organisms may allow for validation of some of the more predictive approaches that apply the physiological niche concept. Combining these approaches may be vital for understanding biological responses to environmental change, which may embody changes to many physical and biological variables (Sagarin and Pauchard, 2010), and can play an important role in informing predictive models of environmental change.

The limited movement of many adult forms of rocky shore species makes small scale acclimation (i.e. over distances of 10s to 100s of metres) more important than in many other marine and terrestrial environments. Indeed, there is considerable variability in stress on rocky shores at the spatial scale of centimetres, with crevices or shade providing refuges from heat and desiccation stress (Chapperon et al., 2013; Denny et al., 2011; Garrity, 1984; Stafford, 2002). Furthermore, at distances of metres (rather than 10s of metres), there can be considerable changes in vertical distribution patterns and the stresses associated with these patterns. For most grazing molluscs, these small scale patterns may be important over short temporal scales (such as tidal cycles), but less important for long-term adaptation than the 10s of metres scale examined here. *C. grata*, for example, is not a homing limpet, and unless crevices are plentiful, is frequently found on flat rock (Williams and Morritt, 1995; Williams et al., 2005). It can displace by several metres each day, but will be confined to short sections of shore separated by sand or water for its adult life. As such, examining a 10 m stretch of shore for a biological variable related to long-term adaptation is logical. However, to assess a shorter term acute response to stress as may occur during the emersion period of a tidal cycle, examining shore characteristics on the scale of centimetres would be more appropriate. For more mobile rocky shore organisms, such as crabs, larger spatial scales, including much wider vertical ranges would need to be considered. On some shores, predation risk also needs careful consideration, as it greatly affects community structure and likely acts as a strong selective pressure. Molluscan predators such as dogwhelks can be quantified in a similar manner to grazers, using quadrat counts; however, these were not seen in the quadrat counts in this study. Fish as predators and grazers can be important on many rocky shores, especially in tropical regions (although less so in Hong Kong; Williams et al., 2000), and if this is an important consideration, then high tide surveys would also need to be conducted.

To examine evolutionary pressures it should be noted that intergenerational dispersal may be higher for many rocky shore (and other marine) species than many terrestrial species such as plants or insects. Therefore, even in terrestrial systems adaptation (through long-term acclimation or evolution) may be localised for many species (e.g. Ayre, 1995 and references within). As such, the ability to calculate stress regimes at local, biologically relevant, scales has considerable implications for successfully predicting organisms' responses to climate change. Rapid in-field measurements, such as those presented here, allow for quantification of differences in stress regime of organisms, and could be used to rapidly assess large numbers of micro- to meso-scale habitats.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ecoinf.2014.11.006.

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