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1 **High thermal stress responses of *Echinolittorina* snails at their**  
2 **range edge predict population vulnerability to future warming**

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20 **Abstract**

21 Populations at the edge of their species' distribution ranges are typically living at the  
22 physiological extreme of the environmental conditions they can tolerate. As a species'

23 response to global change is likely to be largely determined by its physiological  
24 performance, subsequent changes in environmental conditions can profoundly influence  
25 populations at range edges, resulting in range extensions or retractions. To understand the  
26 differential physiological performance among populations at their distribution range edge  
27 and center, we measured levels of mRNA for *heat shock protein 70 (hsp70)* as an indicator  
28 of temperature sensitivity in two high-shore littorinid snails, *Echinolittorina malaccana*  
29 and *E. radiata*, between 1°N to 36°N along the NW Pacific coast. These *Echinolittorina*  
30 snails are extremely heat-tolerant and frequently experience environmental temperatures  
31 in excess of 55°C when emersed. It was assumed that animals exhibiting high temperature  
32 sensitivity will synthesize higher levels of mRNA, which will thus lead to higher energetic  
33 costs for thermal defense. Populations showed significant geographic variation in  
34 temperature sensitivity along their range. Snails at the northern range edge of *E. malaccana*  
35 and southern range edge of *E. radiata* exhibited higher levels of *hsp70* expression than  
36 individuals collected from populations at the center of their respective ranges. The high  
37 levels of *hsp70* mRNA in populations at the edge of a species' distribution range may serve  
38 as an adaptive response to locally stressful thermal environments, suggesting populations  
39 at the edge of their distribution range are potentially more sensitive to future global  
40 warming.

41 **Key words:** biogeography, global change, heat shock response, physiological stress,  
42 distribution range

43

44 **Introduction**

45 Rising atmospheric carbon dioxide levels have led to changes in global temperature,  
46 climate in general and seawater chemistry resulting in subsequent impacts on species'  
47 distribution patterns (Hofmann & Todgham, 2010; Parmesan, 2003). Variation in  
48 environmental conditions resulting from climate change has, for example, been shown to  
49 result in broad biogeographic shifts of species, such as range retraction or expansion (Perry  
50 *et al.*, 2005; Poloczanska *et al.*, 2013), as well as localized changes in species which are  
51 distributed in spatially patchy habitats (Somero, 2012). As many species occur over wide  
52 latitudinal or altitudinal gradients, populations in different locations of a species'  
53 distribution range may experience very different environmental challenges (Buckley &  
54 Huey, 2016; Mathiasen & Premoli, 2016; Vergeer & Kunin, 2013). Typically, populations  
55 at the edge of a species' range are located at the extreme of the environmental stress  
56 gradient that the species can tolerate (Gaston, 2009; Mägi *et al.*, 2011; Sexton *et al.*, 2009).  
57 Such populations are, therefore, assumed to be constrained by current environmental  
58 conditions and subsequently are predicted to be more sensitive to future climate change  
59 (Sagarin & Somero, 2006; Sorte & Hofmann, 2004). Understanding the divergent  
60 responses between different populations along their distributional range is, therefore,  
61 important to inform predictions of the ecological impacts of climate change.

62 Physiological adaptation is a key response for species to cope with environmental  
63 variations, and the physiological responses of different populations are closely related to  
64 their local environments (Helmuth, 2009; Seabra *et al.*, 2015; Somero *et al.*, 2016). The  
65 heat shock response (HSR), which is highly conserved across almost all taxa (Feder &  
66 Hofmann, 1999; Somero *et al.*, 2017), is a homeostatic response that maintains the correct  
67 protein-folding environment in the cell (Guisbert *et al.* 2008) and, as such, heat shock

68 proteins (Hsps) are considered to be one of the most important cellular defense mechanisms  
69 against environmental stresses (Sørensen *et al.*, 2003; Tomanek, 2010). As a consequence,  
70 Hsp expression is believed to reflect both the environmental variability associated with  
71 different microhabitats and its interaction with large-scale environmental gradients across  
72 the whole distribution range of a species (Dutton & Hofmann, 2009; Lima *et al.*, 2016;  
73 Sagarin & Somero, 2006, Wang *et al.*, 2018). Environmental stress is also an evolutionary  
74 driver for local adaptation in natural populations (Kawecki & Ebert, 2004). Generally,  
75 natural selection acts on the HSR in different populations by changing the expression of  
76 *hsp* genes, rather than by gene mutations that affect the properties of the heat shock proteins  
77 themselves (Bettencourt *et al.*, 2002; Dutton & Hofmann, 2009; Sagarin & Somero, 2006;  
78 Sorte & Hofmann, 2004). As a result, geographic variation of expression of Hsps as well  
79 as plasticity of expression are both heritable (Somero *et al.*, 2017), and consequently,  
80 divergent HSRs to local thermal environments have been identified in various marine  
81 invertebrates (Gleason and Burton, 2015; Schoville *et al.*, 2012), showing the evolutionary  
82 adaptation of the HSR to local environmental conditions (Tedeschi *et al.*, 2016) and its  
83 suitability to be used as a measure of thermal sensitivity (Tomanek & Somero, 1999a).

84 Rocky shores are one of the most physically challenging habitats on earth, both on a  
85 local scale (as a result of tidal changes; wave action etc. Helmuth and Hofmann, 2001;  
86 Little *et al.*, 2009) but also with highly variable thermal environments over biogeographic  
87 scales (Harley, 2008; Helmuth *et al.* 2002). Activation of the HSR within the typical range  
88 of body temperatures organisms experience is part of the strategy of intertidal species to  
89 cope with thermal stress. Distinct strategies of Hsp70 expression can be employed by  
90 intertidal species occupying different heights along the intertidal zone. High-intertidal

91 species, for example, tend to have higher constitutive levels of Hsp70 than low- and mid-  
92 intertidal species. By contrast, lower-occurring species maintain low levels of Hsp70, but  
93 are capable of rapidly inducing high levels of synthesis when exposed to heat stress (Dong  
94 *et al.*, 2008; Nakano & Iwama, 2002). Different populations of widely distributed species,  
95 thus, can face divergent thermal regimes at different locations along their distribution range  
96 (Helmuth *et al.* 2002, Dong *et al.* 2017). As a result, persistent regional differences in tidal  
97 regimes, climate and other environmental factors will act as selective forces to influence  
98 the physiology of intertidal species which span broad latitudinal ranges. Upper limits of  
99 the temperature range of Hsp synthesis are close to the highest body temperatures that these  
100 organisms experience under natural conditions (Sorte & Hofmann, 2005; Tomanek &  
101 Somero, 1999; Tomanek, 2002). Consequently, Hsp synthesis may be a thermally sensitive  
102 weak-link that contributes to setting species' thermal tolerance limits (Somero, 2004). The  
103 HSR is thus a convenient biochemical indicator to assess levels of physiological stress  
104 among populations along their distribution range (Dutton & Hofmann, 2009; Sagarin &  
105 Somero, 2006; Sorte & Hofmann, 2004, Stillman & Tagmount, 2009).

106 At present, many intertidal organisms experience temperatures at or above their  
107 tolerance limits during low tides in stressful seasons (Wolcott, 1973; Williams 1994;  
108 Williams *et al.*, 2005; Somero, 2012; Zhang *et al.*, 2016). High shore species suffer from  
109 the most extreme thermal stress and, as a result, have been postulated to be especially  
110 vulnerable to climate warming (Tomanek & Somero, 1999b; Somero, 2012). The littorinid  
111 snails, *Echinolittorina malaccana* and *E. radiata* are widely distributed along the NW  
112 Pacific (NWP) coast, with representatives of the two species covering over 50 degrees of  
113 latitude from 5°S to 45°N-(Fig. 1, Reid, 2007). Living on the high shore, littorinid snails

114 experience long emersion periods when environmental (=rock surface) temperatures can  
115 exceed 60 °C (Williams, 1994; Marshall *et al.*, 2011; Seuront & Ng, 2016). The thermal  
116 environment of intertidal species along the NWP coastline is extremely variable among  
117 different locations (Dong *et al.*, 2015), and shows a highly non-linear relationship with  
118 latitude (Helmuth *et al.*, 2002; Lima *et al.*, 2016; Dong *et al.*, 2017). Different populations  
119 of *Echinolittorina* snails, therefore, face extreme and divergent thermal stresses along their  
120 distribution ranges.

121 Using specifically designed primers, we can precisely detect the mRNA levels of  
122 inducible isoforms of Hsp70 (*hsp70*), which is an important biochemical indicator to assess  
123 levels of physiological stress. Up-regulation of *hsp70* in response to thermal stress has been  
124 well documented in a variety of marine taxa (bivalves, gastropods and shrimps; Clark *et al.*,  
125 2008; Cottin *et al.*, 2010; Giomi *et al.*, 2016; Han *et al.*, 2013; Prusina *et al.*, 2014). In  
126 a previous study, we found that Hsp70 protein levels significantly increased in response to  
127 thermal stress in *Echinolittorina malaccana* (Marshall *et al.*, 2011) which supports the use  
128 of up-regulation of *hsp70* mRNA to predict the production of Hsp70 protein in  
129 *Echinolittorina* snails, and the positive relationship between levels of *hsp70* mRNA and  
130 Hsp70 protein. The present study was, therefore, designed to highlight the importance of  
131 estimating population-specific physiological performance when evaluating and predicting  
132 the ecological impacts of climate change on species with wide geographic distributions.  
133 Specifically, we investigated variation in heat shock response using levels of mRNA for  
134 *hsp70* to test the hypothesis that populations at the range edge of the two *Echinolittorina*  
135 snails would be more sensitive to thermal stress than those located towards the center of  
136 their distribution.

## 137 **Materials and Methods**

### 138 **Expression of heat shock protein in field-acclimatized populations across the species'** 139 **biogeographic ranges**

140 The latitudinal distribution of *Echinolittorina malaccana* ranges from India through  
141 Southeast Asia and into China, whilst *E. radiata* is a more northern species and ranges  
142 from Japan to Vietnam (Reid, 2007, Fig. 1). Based on the distribution of the two species in  
143 different ecoregions and previous phylogeographic studies (Reid, 2007; Spalding et al.,  
144 2007; Dong *et al.*, 2012; Wang *et al.*, 2015, 2016), the range center and edge of each species  
145 were classified. In the present study, the distribution center for *E. malaccana* includes the  
146 ecoregions of the Java Sea, Malacca Strait, Gulf of Thailand, Southern Vietnam and Gulf  
147 of Tonkin, and its northern distribution range includes the two north most ecoregions (Fig.  
148 1); whilst for *E. radiata*, its distribution center includes the two ecoregions in the East  
149 China sea and Yellow Sea, and its southern distribution edge include two south most  
150 ecoregions (Fig. 1).

151 To measure expression of *hsp 70* under natural conditions (field-acclimated), ten  
152 locations along the species' ranges were selected, including for *E. malaccana* populations:  
153 Sanya (18.17°N), Da Nang (16.07°N), Si Chang (13.15°N), Sepang (2.59°N) and  
154 Singapore (1.29°N) which were determined as central populations, while Keelung  
155 (25.14°N), Xiamen (24.42°N) and Hong Kong (22.21°N) were determined as northern  
156 edge populations; and for *E. radiata*: Qingdao (36.05°N), Zhoushan (29.94°N) and  
157 Keelung (25.14°N) which were determined as central populations, while Xiamen  
158 (24.42°N) and Hong Kong (22.21°N) populations were determined as southern edge



159 populations (Fig. 1). Daily maximal air temperature (2012-2014) for each site was  
160 download from NASA and analyzed in R using *ncdf4* package (Bosilovich *et al.*, 2015).  
161 The thermal environment along the Northwestern Pacific coastline shows a highly non-  
162 linear relationship with latitude (Fig. S1), with highest air temperatures at Si Chang  
163 (36.78°C) and Zhoushan (36.87°C). The highest temperatures of the year, which represent  
164 the most stressful season, however, occur in July to September for each location (Fig. S1).  
165 To avoid the confounding effects of seasonal acclimatization on *hsp70* expression levels  
166 from animals sampled from the shore (Buckley *et al.*, 2001; Sagarin and Somero, 2006),  
167 samples were collected during the season of highest environmental temperatures (the most  
168 thermally stressful = hot season) from July to September in 2012-2014 (Table 1). To  
169 minimize the effects variation of *hsp70* levels across tidal cycles (Gracey *et al.*, 2008) and  
170 the interaction of stress from temperature and rainfall (Dong *et al.*, 2014), 15-20 individuals  
171 were sampled from two semi-exposed rocky shores sites (sites A and B, separated by at  
172 least 30 meters and distant from obvious anthropogenic influences) in each location (except  
173 Keelung) during low spring tides on days with no rain. On collection, body temperatures  
174 were immediately measured by inserting a thermocouple past the operculum onto the foot  
175 (K-type thermocouple, Lutron thermometer  $\pm 0.1^\circ\text{C}$ ) and then shells were immediately  
176 cracked and immersed in RNAlater (Life Technologies, Carlsbad, CA, USA). All samples  
177 were sent to the State Key Laboratory of Marine Environmental Science (MEL), Xiamen  
178 University and kept at  $-80^\circ\text{C}$  until analysis.

### 179 **Laboratory assessment of heat shock protein gene expression**

180 To determine whether there are divergent heat shock responses among populations,  
181 snails were collected from Xiamen (*Echinolittorina malaccana* and *E. radiata*), Hong

182 Kong (*E. malaccana* and *E. radiata*) and Sanya (*E. malaccana*). At each location, 150  
183 individuals (7-8 mm maximal shell length) were collected from the two sites and taken  
184 back to the laboratory during the most stressful season (as above). In the laboratory,  
185 animals were sprayed with filtered seawater and kept at 28 °C for three days for short-term  
186 acclimation to reduce the effects of recent thermal history. Five snails were put into small  
187 vials in air ( $\Phi = 22$  mm, height = 35 mm), which were placed into a Grant water bath (Grant  
188 Instruments, Cambridge, UK). Temperature within a blank vial in the water bath was  
189 recorded every 1 min using a Fluke digital recording thermometer and a fine K-type  
190 thermocouple (Lutron, Taiwan). Temperatures were increased from 28 to 50 °C at a rate  
191 of 5°C/hr as described in Marshall *et al* (2011). When the temperature reached 50 °C, it  
192 was increased to 57 °C at a rate of 2°C/hr. Three randomly chosen vials were removed at  
193 35, 40, 45, 47, 49, 51, 53, 55 and 57 °C ( $\Sigma n = 9$  temperatures x 3 replicates = 27). After  
194 heating, the animals in each vial were returned to 28°C seawater for 2hrs, and then placed  
195 in RNAlater after cracking their shells. Samples collected at Hong Kong and Sanya were  
196 treated at The Swire Institute of Marine Science (SWIMS), The University of Hong Kong;  
197 while samples collected at Xiamen were treated at MEL, Xiamen University. All samples  
198 were sent to MEL to analyze gene expression of *hsp70*.

### 199 **Quantitative analysis of Hsp70 mRNA**

200 Total RNA was isolated from ~30 mg of foot tissue from each individual using an  
201 Eastep™ Universal RNA Extraction Kit (Promega, Madison, WI, USA) and quantified  
202 using a NanoDrop ND-1000 photometer (Thermo Fisher Scientific, Waltham, MA, USA).  
203 A sample of 0.5 µg of total RNA was used as the template for synthesis of the first strand  
204 of cDNA using a PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Otsu, Shiga,

205 Japan). The real-time PCR Primers were designed based on the partial sequences of four  
206 genes which cloned from *E. malaccana*: *hsp 70* (GenBank accession No. KR082142), *beta-*  
207 *actin* (GenBank accession No. KR082141), *beta-tubulin* (GenBank accession No.  
208 KR082140) and *elongation factor 1-gamma* (GenBank accession No. KR082139) using  
209 Beacon Designer 7 software (Premier Biosoft International, Palo Alto, CA, USA). The  
210 efficiency of each primer set for *E malaccana* and *E. radiata* was determined by real-time  
211 PCR with an appropriate dilution series of cDNA prior to sample analyses. The sequences  
212 of primers, the product length and the efficiency for each species are shown in Table S1.  
213 Real-time PCR was carried out on a CFX96 Touch Real-Time PCR System (Bio Rad,  
214 Hercules, CA, USA) in a 20- $\mu$ l reaction volume containing 10  $\mu$ l of 2 $\times$  master mix  
215 (DyNAmo Flash SYBR Green qPCR Kit, Thermo Fisher Scientific, Waltham, MA, USA),  
216 1  $\mu$ l of each primer (10 nmol  $\mu$ l<sup>-1</sup>), 1  $\mu$ l of cDNA template and 7  $\mu$ l of RNase-free water.  
217 PCR conditions were as follows: 95 °C 7 min; 40 cycles of 95 °C 20 s, 60 °C 1 min; and a  
218 final melt curve step. All samples were measured in triplicate.

## 219 **Data processing and analysis**

220 *Hsp70* mRNA relative expression data were analyzed using Bio-Rad CFX Manager  
221 3.1 software (Bio-Rad, Hercules, CA, USA). Three genes (*beta-actin*, *beta-tubulin* and  
222 *elongation factor 1-gamma*) were selected as the reference genes. One field sample,  
223 collected at Da Nang, was randomly selected as the reference sample.

224 For all analyses, generalized linear models (GLM) were performed using a gamma error  
225 distribution, following Friedman *et al.* (2010). To illustrate the latitudinal pattern of *hsp70*  
226 expression levels, latitude was the explanatory variable. Residuals from the GLM varied

227 between sites in some locations, indicating potential spatial variations at a smaller scale  
228 than latitude ( $P > 0.05$  in all cases except for Da Nang and Sepang for *E. malaccana*, and  
229 Xiamen and Qingdao for *E. radiata*, where  $P$  ranged from 0.03 to  $< 0.001$ ). Data from the  
230 two sites at each location, however, were pooled as there were too few levels to accurately  
231 estimate random (i.e. between sites) effects (Bolker 2015).

232 To investigate if the temperature sensitivity of *hsp70* expression (change in *hsp70*  
233 expression per unit change in body temperature) varied within the littorinids' range,  
234 distribution location (range edge, RE or range center, RC), body temperature, and their  
235 interactions were used as explanatory variables. Such a geographical pattern was further  
236 analyzed by testing if the temperature sensitivity of *hsp70* expression in both  
237 *Echinolittorina malaccana* and *E. radiata* varied with latitude within their respective RE  
238 or RC with, again, body temperature, latitude and their interactions as explanatory  
239 variables.

240 In the laboratory assessment, *hsp70* mRNA production in both *E. malaccana* and *E.*  
241 *radiata* was investigated to test if *hsp70* expression differed between temperatures,  
242 locations, and their interactions. Data from the two sites at each location were again pooled  
243 (see above) as residuals from the GLM generally did not vary between sites ( $P > 0.05$  in  
244 all cases except in Xiamen for *E. radiata*, where  $P = 0.02$ ). All tests were performed with  
245 the base package and *car* (Fox *et al.*, 2013) package in R (version 3.3.1, R Core Team,  
246 2016).

## 247 **Results**

248 Body temperature and corresponding *hsp70* levels for each individual are reported in  
249 Table S1. On a broad geographical scale, *hsp70* expression levels showed clear latitudinal  
250 patterns, increasing with latitude in *Echinolittorina malaccana* ( $t_{235} = 6.18$ ,  $P < 0.001$ ) and  
251 decreasing in *E. radiata* ( $t_{169} = -4.84$ ,  $P < 0.001$ , Fig. 2). Levels of *hsp70* expression also  
252 showed a positive relationship with field body temperature in both snails (Table 2).

253 When populations were divided according to their location into range edge (RE) and  
254 range center (RC) groups, *hsp70* mRNA levels increased with field body temperature in  
255 both RE and RC for *E. malaccana* (Table 2, Fig. 3a). In the *E. malaccana* RC group, *hsp70*  
256 expression of snails increased with field body temperature ( $\chi^2_1 = 4.370$ ,  $P = 0.037$ ) but  
257 varied among different locations ( $\chi^2_4 = 31.750$ ,  $P < 0.001$ ). Similarly, *hsp70* expression of  
258 snails increased with field body temperature ( $\chi^2_1 = 34.474$ ,  $P < 0.001$ ) and varied among  
259 different locations ( $\chi^2_2 = 8.294$ ,  $P = 0.016$ ) in the *E. malaccana* RE group. The temperature  
260 sensitivity (changes in *hsp70* expression per unit change in body temperature) was higher  
261 in RE than RC (RE:  $t_{83} = 6.077$ ,  $P < 0.001$ ; RC:  $t_{138} = 2.099$ ,  $P = 0.038$ ).

262 *Hsp70* mRNA levels also increased with field body temperature in the RE but not in the  
263 RC group for *E. radiata*, (Table 2, Fig. 3b). In contrast to *E. malaccana*, in the *E. radiata*  
264 RC group, *hsp70* expression did not change with field body temperature ( $\chi^2_1 = 0.229$ ,  $P =$   
265  $0.632$ ) but, similar to *E. malaccana*, did vary among different locations ( $\chi^2_2 = 42.982$ ,  $P <$   
266  $0.001$ ). In the *E. radiata* RE group, however, *hsp70* levels increased with field body  
267 temperature ( $\chi^2_1 = 42.951$ ,  $P < 0.001$ ) and varied among locations ( $\chi^2_1 = 9.806$ ,  $P = 0.002$ ),  
268 as with *E. malaccana*, suggesting that temperature sensitivity of *hsp70* expression was  
269 higher at the distribution RE for both species. The mean *hsp70* expression level was also

270 higher at the RE for both *E. malaccana* ( $t=-8.418$ ,  $df=98.658$ ,  $P < 0.001$ ) and *E. radiata* ( $t$   
271  $= -3.610$ ,  $df = 88.549$ ,  $P < 0.001$ , Fig. 4).

272 In the laboratory assessments, *hsp70* expression increased with increasing snail body  
273 temperature (Table 3, Fig. 5). Relative *hsp70* expressions in *E. malaccana* from Hong  
274 Kong and Sanya were higher than those of Xiamen individuals when body temperatures  
275 were higher than 47 °C (Fig. 5). The levels of *hsp70* in *E. radiata* from Hong Kong were  
276 higher than those of snails from Xiamen at body temperatures less than 45 °C, but were  
277 similar or slightly higher at body temperatures higher than 45 °C (Table 3).

## 278 Discussion

279 Individuals collected from populations at the northern range of *Echinolittorina*  
280 *malaccana* and the southern range of *E. radiata* had higher *hsp70* levels than individuals  
281 collected from the respective species' range centers. The temperature sensitivity of *hsp70*  
282 expression was also dramatically higher in the range edge populations than in those at the  
283 species' range centers. Whilst heat shock proteins can effectively repair heat-induced  
284 protein damage, energy expenditure will be required at several stages in the HSR (Somero,  
285 2002). The strong HSR recorded for populations at the species' range edges, therefore,  
286 implies an important diversion of energy allocation, which ultimately may influence  
287 ecological fitness.

288 *CHECK letter for changes Echinolittorina* snails can depress their resting metabolism  
289 within a thermally insensitive range (approximately 35-46°C) while experiencing high  
290 temperatures, which enhances their thermal tolerance (Marshall *et al.*, 2010; Marshall *et*  
291 *al.*, 2011). Within this thermal insensitive range, metabolic rate is delinked from increasing

292 temperature, which is in contrast to the predictions of the UTD model (universal  
293 temperature-dependence model; Clarke, 2004). When temperature exceeds the upper  
294 thermal threshold for thermal insensitivity (approximately 46 °C), however, the  
295 metabolism of snails is elevated to meet the energy demands of maintenance at these  
296 temperatures (Marshall *et al.*, 2011). Thus, metabolic rate returns to be positively  
297 correlated with temperature. Metabolic depression may, therefore, influence the production  
298 of Hsp70. over a broad thermal range. Marshall and co-workers, for example, showed that  
299 levels of Hsp70 initially increased with increasing temperature, but then reached a plateau  
300 during the thermally insensitive range, but finally increased when temperature exceeded  
301 the upper limit of the thermally insensitive temperature (Marshall *et al.*, 2011, Fig. 7). As  
302 a consequence of this metabolic depression, these snails can save energy when in their  
303 thermally insensitive zone, which may allow them to conserve energy for increased  
304 production of Hsp70 at higher temperature, leading to enhanced thermal tolerance. The  
305 structural and functional cellular modifications associated with this depression are,  
306 however, still unclear, and the role of latent mRNA remains to be clarified. Snails are  
307 killed, however, when they are exposed to temperatures beyond their thermal insensitivity  
308 threshold for long durations, suggesting a threshold above which the rate of energy demand  
309 exceeds the capacity for cellular energy generation. The time to gain energy is severely  
310 constrained on the shore to periods when awash by the rising or falling tide. When  
311 resources are limiting, any investment in a specific trait must carry a cost that is expressed  
312 in trade-offs with other traits (Araújo *et al.*, 2015). A trade-off between the production of  
313 defense metabolites and growth has, for example, been clearly demonstrated in ectothermic  
314 animals, plants and algae (Clobert *et al.*, 2000; Sparkman & Palacios, 2009; Dworjanyn *et*

315 *al.*, 2006; Nylund *et al.*, 2013). The fitness of range edge populations, therefore, is likely  
316 to be reduced by shifting investment from growth, storage, reproduction to maintenance  
317 (Jump & Woodward, 2003; Viejo *et al.* 2011; Sokolova *et al.*, 2012), which would increase  
318 the vulnerability of such populations to future changes in climate.

319 Stronger stress responses at a species' distribution range edge have rarely been recorded  
320 in rocky shore species due to the complex mosaic of thermal environments experienced in  
321 the intertidal zone (Helmuth *et al.*, 2002). Sagarin & Somero (2006), for instance, showed  
322 that the patterns of expression of Hsp70 in the mussel, *Mytilus californianus*, and whelk,  
323 *Nucella ostrina*, were complex and more consistent with the surrounding, local,  
324 environmental conditions than with broader latitudinal effects. Lima *et al* (2016) also found  
325 that Hsp70 levels in the limpet, *Patella vulgata*, were tightly associated with immediate  
326 microtopography rather than with shore latitude. In the present study, however, the *hsp70*  
327 expression levels in *E. malaccana* and *E. radiata* showed clear latitudinal patterns at the  
328 broad geographical scale, suggesting that strong latitudinal gradients in environmental  
329 stressors can drive divergent responses among populations throughout their geographic  
330 ranges. Similarly, Sorte & Hofmann (2004) found that the dogwhelk, *Nucella canaliculata*,  
331 living near their southern range edge also had higher levels of Hsp70 than those near the  
332 center of their range. Pearson and co-worker (2009) also found that expression of heat  
333 shock genes was higher at the same temperature in range edge populations as compared to  
334 populations closer to the center of the range of the alga, *Fucus serratus*.

335 The vertical distribution (Ferreira *et al.* 2014), ability to acclimate (Stillman 2003) or  
336 mobility (which allows species to use behavioural thermoregulation, Marshall *et al.*, 2013;  
337 Ng *et al.* 2017) may explain species-specific differences in stress response at species' range



338 edge and centers. It has been hypothesized that under future global warming, extinction  
339 risks are generally expected to be highest at species' range edges for organisms that exhibit  
340 differential stress responses and stronger responses at distribution range edges (Sexton *et*  
341 *al.*, 2009). In contrast, mortality will be more likely at some 'hot spots' for some intertidal  
342 organisms which don't exhibit linear stress response along the latitude (Sagarin & Somero,  
343 2006). Thus, species-specific differences in individual performance at range edges and  
344 range centers are important to consider when predicting the impacts of climate change on  
345 the potential distribution range shifts of intertidal organisms.

346 Temperature is a main inducing factor for the expression of *hsp* genes. In the present  
347 study, there was no significant variation in the body temperatures of *Echinolittorina*  
348 *radiata* individuals between range edge and center populations. Thus, the higher *hsp70*  
349 levels of *E. radiata* were mainly the result of higher temperature sensitivity of HSR of the  
350 range edge populations. In the case of *E. malaccana*, the body temperatures of samples  
351 collected from the range edge populations were relatively higher than those from the range  
352 center which, together with their stronger thermal sensitivity, should contribute to the  
353 higher *hsp70* levels recorded at the range edge.

354 The higher temperature sensitivity of *hsp70* expression may be caused by plasticity  
355 and/or evolutionary adaptation to the local thermal environment at the edge of the species'  
356 distribution range. Range edge populations are assumed to be physiologically constrained  
357 (Sorte & Hofmann, 2004), and thus to suffer selective pressures to develop novel strategies  
358 for persistence and adaptation at these challenging environments to avoid population  
359 extinction (Araújo *et al.* 2015). In the laboratory assessments, the expression patterns of  
360 *hsp70* in response to heat stress also showed significant variations among different

361 populations, suggesting plasticity and/or local adaptation of HSR in response to heat stress.  
362 *hsp70* was more readily induced in RC populations (Sanya) than RE populations (Hong  
363 Kong and Xiamen). It is likely that thermal regimes in RE locations may have already  
364 induced higher levels of Hsps in the cell which would be sufficient to tolerate mild  
365 temperature stress (Buckley *et al.*, 2001). Local adaptation can result in resident genotypes  
366 that have a higher fitness in their native habitat than foreign genotypes from more distant  
367 populations (Kawecki and Ebert, 2004; Sanford and Kelly, 2011). Given the present  
368 approach, we are unable to determine whether the higher temperature sensitivity of *hsp70*  
369 expression between the range edge and center populations was genetically fixed. If,  
370 however, the variation of HSR between the range edge and the center populations was due  
371 to local adaptation, any immigration from the range center to the range edge will be  
372 unlikely to be successful, because the range center populations will lack the ability to  
373 achieve the required higher *hsp70* levels in response to thermal stress experienced at the  
374 range edge.

375 Lastly, we recognize that the use of a single stress indicator, *hsp70* mRNA, provides  
376 only a partial image of heat stress in field populations. Different stress-related genes have  
377 different onset temperatures for expression, so including a battery of stress-responsive  
378 genes in future studies would provide further insights into the variation among populations  
379 in capacities for responding to heat stress. Another focus of future studies could involve  
380 time-dependent changes in mRNAs of stress-related proteins, as turnover of mRNA,  
381 including *hsp70* mRNA, is rapid and, therefore, the time between field heat stress and  
382 sampling of specimens can be critical in experimental design (see Gracey *et al.*, 2008).

383 Obtaining samples across a time period that spans the full tidal cycle would be a logical  
384 and insightful next step in such analyses.

## 385 **Conclusions**

386 The populations that inhabit the edge of a species' distributional range are likely to be  
387 critically important in determining a species' response to ongoing climate change. Using  
388 *hsp70* expression as an indicator, our results showed significant geographical variation in  
389 physiological response of two high shore *Echinolittorina* snails, suggesting the adaptive  
390 differentiation of populations to local environmental conditions. The temperature  
391 sensitivity of *hsp70* expression was higher in range edge than range center populations,  
392 and the individuals collected at the northern range edge of *E. malaccana* and southern range  
393 edge of *E. radiata* had higher *hsp70* expression than individuals collected from their  
394 respective range center. The high inducible *hsp70* levels in the range edge populations  
395 likely serve as an adaptive response to the stressful thermal environment at the expense of  
396 energy allocation to other fitness traits, and as such populations living at their species'  
397 range edges are likely to be more vulnerable to future changes in environmental conditions.

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### 407 **Author Contributions**

408 Y.-W.D and G.A.W designed the study, and G.-D.H, S.R.C, M.G, B.K.K.C, K.A.A.A, N.H and  
409 J.W performed the research. G.-D.H and T.Y.H analyzed the data, and G.-D.H, Y.-W.D and G.A.W  
410 wrote the paper.

### 411 **Conflict of Interest**

412 The authors have no conflict of interest to declare.

413

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641

642 **Tables**

643 Table 1. Geographic locations, range distribution of species' populations and sampling  
 644 dates for *E. malaccana* and *E. radiata*. The ecoregions of different population located were  
 645 showed as Spalding et al. (2007) described.

Locations	Ecoregion	Distribution: Range edge/center (RE/RC)	Sampling dates	Latitude
<i>Echinolittorina malaccana</i>				
Keelung, Taiwan	52	RE	July 3-4, 2012	25.14° N
Xiamen, Mainland China	113	RE	September 11, 2012	24.42° N
Hong Kong	113	RE	August 23-24, 2012	22.21° N
Sanya, Mainland China	112	RC	August 20, 2013	18.17° N
Da Nang, Vietnam	112	RC	September 12, 2013	16.07° N
Si Chang Island, Thailand	115	RC	September, 2014	13.15° N
Selangor, Malaysia	117	RC	September 10, 2014	2.59° N
Singapore	117	RC	September, 2014	1.29° N
Locations	Range distribution		Sampling dates	Latitude
<i>Echinolittorina radiata</i>				
Qingdao, Mainland China	50	RC	August 30, 2012	36.05° N
Zhoushan, Mainland China	52	RC	August 17, 2012	29.94° N
Keelung, Taiwan	52	RC	July 3-4, 2012	25.14° N
Xiamen, Mainland China	113	RE	September 11, 2012	24.42° N
Hong Kong	113	RE	August 23-24, 2012	22.21° N

646

647 Table 2. Analysis of deviance for GLM with gamma error distribution, to investigate the  
 648 effects of distribution ranges (range edge, RE or range center RC) and on-shore body  
 649 temperature on the relative *hsp70* expressions in *E. malaccana* and *E. radiata*. n = 148  
 650 for *E. malaccana* RC and n = 89 RE, while n = 100 for *E. radiata* RC and n = 71 RE.

<i>E. malaccana</i>			
Source of variation	$\chi^2$	df	<i>P</i>
Distribution range	11.411	1	< 0.001
Body temperature	32.699	1	< 0.001
Distribution range $\times$ Body temperature	0.011	1	0.915
<i>E. radiata</i>			
Source of variation	$\chi^2$	df	<i>P</i>
Distribution range	5.436	1	0.020
Body temperature	16.591	1	< 0.001
Distribution range $\times$ Body temperature	7.331	1	0.007

651

652 Table 3. Analysis of deviance for GLM with gamma error distribution, to investigate the  
 653 effects of locations (Hong Kong (HK), Sanya (SY) and Xiamen (XM)) and treatment  
 654 temperature on the relative *hsp70* expressions in *E. malaccana* and *E. radiata*. n = 5 – 6 for  
 655 both *E. malaccana* and *E. radiata*. Significant interactions were investigated further using  
 656 GLM at the respective body temperatures.

Source of variation	<i>E. malaccana</i>			<i>E. radiata</i>		
	$\chi^2$	df	<i>P</i>	$\chi^2$	df	<i>P</i>
Location	73.2	2	< 0.001	84.9	1	< 0.001
Treatment temperature	326.4	8	< 0.001	611.3	8	< 0.001
Location × Treatment temperature	145.2	16	< 0.001	105.5	8	< 0.001

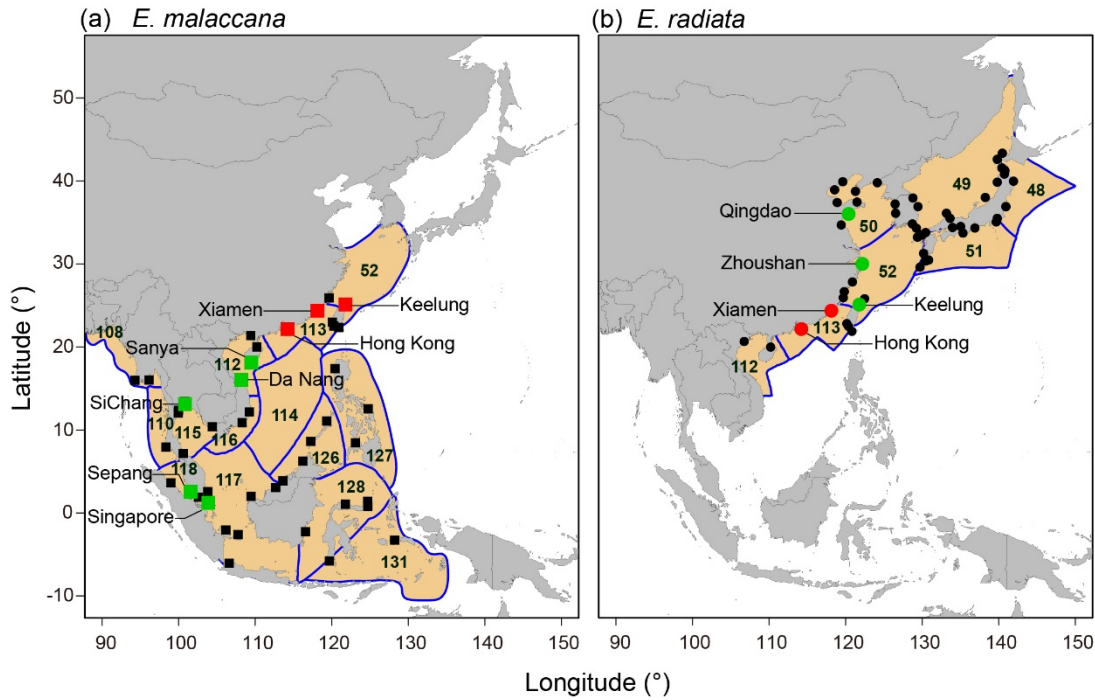
  

Interactions between Location and Treatment temperature		
Treatment temperature(°C)	<i>E. malaccana</i>	<i>E. radiata</i>
35	HK < SY < XM	HK < XM
40	XM = HK = SY	HK < XM
45	XM = HK = SY	HK < XM
47	XM = HK < SY	HK = XM
49	XM < HK < SY	HK = XM
51	XM < HK < SY	XM < HK
53	XM < HK = SY	XM = HK
55	XM < HK = SY	XM = HK
57	XM < HK = SY	XM < HK

657

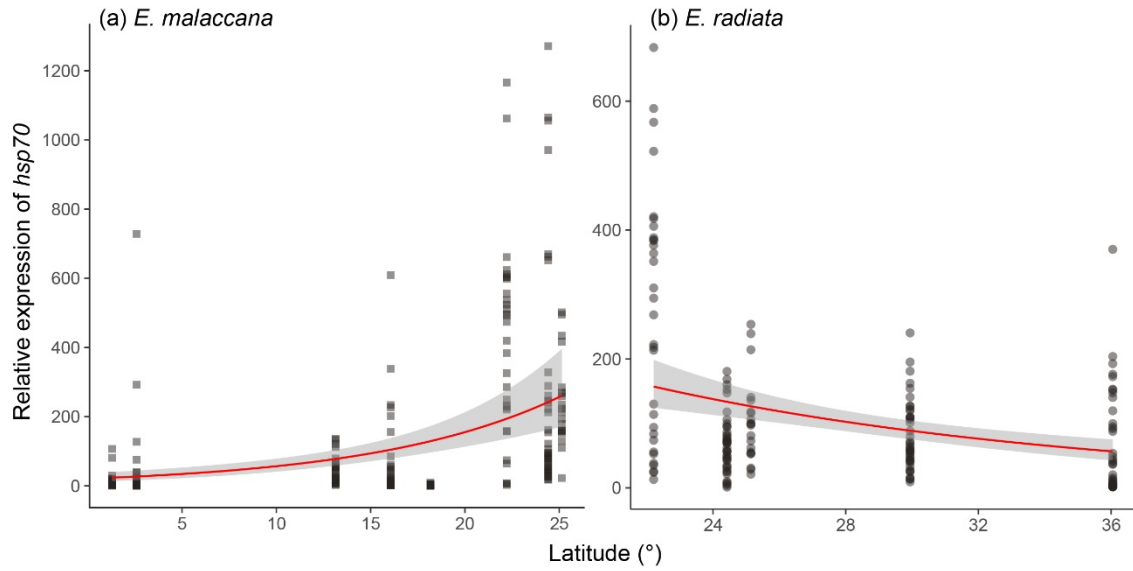


658 **Figures and captions**



659

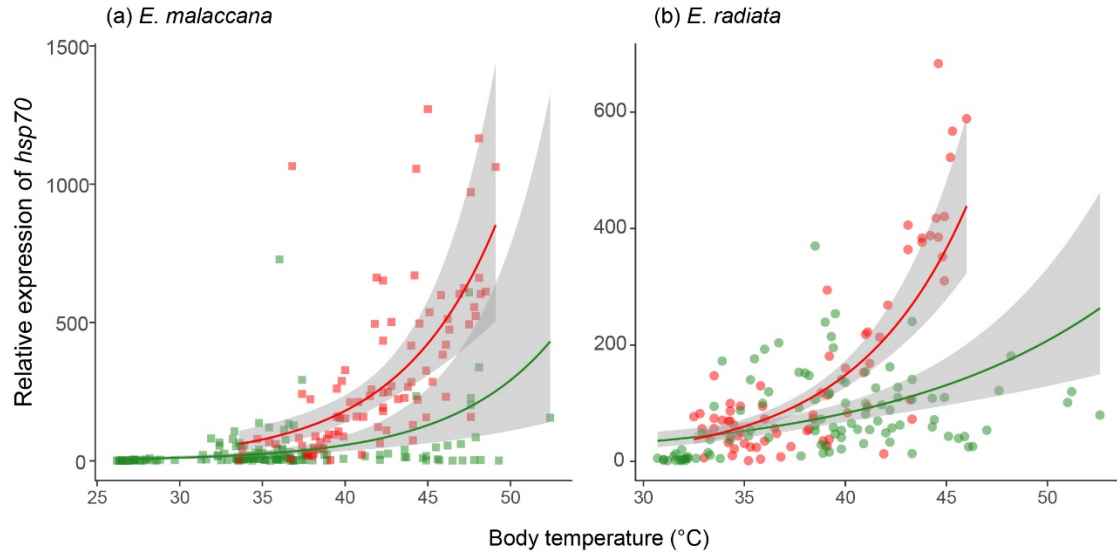
660 Figure 1. Map of the sampling locations for *E. malaccana* (a) and *E. radiata* (b) (for further  
 661 details, see Table 1). Black squares and dots represent the distribution locations for *E.*  
 662 *malaccana* and *E. radiata*, respectively (Reid 2007). Green symbols are sampling locations  
 663 for populations at the species' range center, while the red symbols are sampling locations  
 664 of populations at the species' range edge. The Arabic numerals represent the marine  
 665 ecoregions where the snails distribute: 48, Northeastern Honshu; 49, Sea of Japan; 50,  
 666 Yellow Sea; 51, Central Kuroshio Current; 52, East China Sea; 108, North Bay of Bengal;  
 667 110, Andaman Sea Coral Coast; 112, Gulf of Tonkin; 113, Southern China; 114, South  
 668 China Sea Oceanic Islands; 115, Gulf of Thailand; 116, Southern Vietnam; 117, Java Sea;  
 669 118, Malacca Strait; 126, Palawan; 127, Eastern Philippines; 128, Sulawesi Sea; 131,  
 670 Banda Sea (Spalding *et al.*, 2007).



671

672 Figure 2. Relative expression of *hsp70* from individuals collected on the shore. The scatter  
 673 diagrams show latitudinal patterns of *hsp70* levels of *E. malaccana* (a) and *E. radiata* (b)  
 674 along the Northwestern Pacific coast. The red solid lines represent the generalized linear  
 675 model regression quantile estimate for the latitude as a function of the body temperature or  
 676 *hsp70* levels. The grey areas represent 95% confidence intervals.

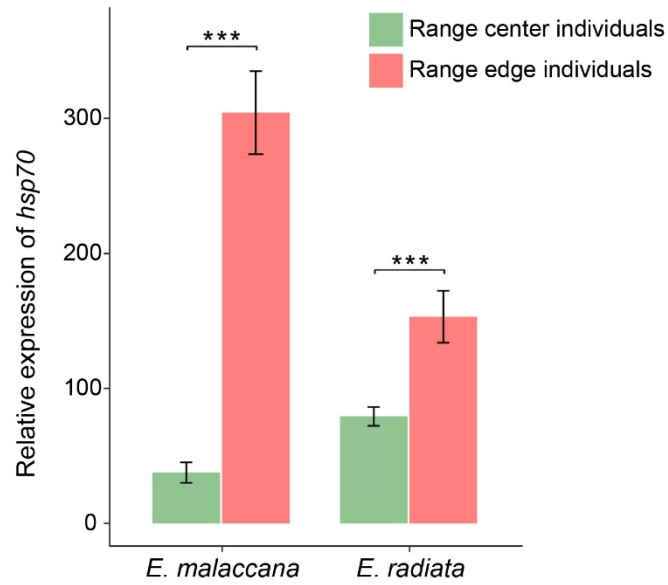
677



678

679 Figure 3. On-shore relative *hsp70* expression of (a) *E. malaccana* and (b) *E. radiata* at  
 680 various body temperatures. Lines represent best-fitting GLM models with gamma error  
 681 distribution, while shaded regions represent 95% confidence intervals. Red lines and points  
 682 represent data from snails at their range edge, while green lines and points represent snails  
 683 at their range center.  $n = 148$  for *E. malaccana* RC and  $n = 89$  RE, while  $n = 100$  for *E.*  
 684 *radiata* RC and  $n = 71$  RE.

685

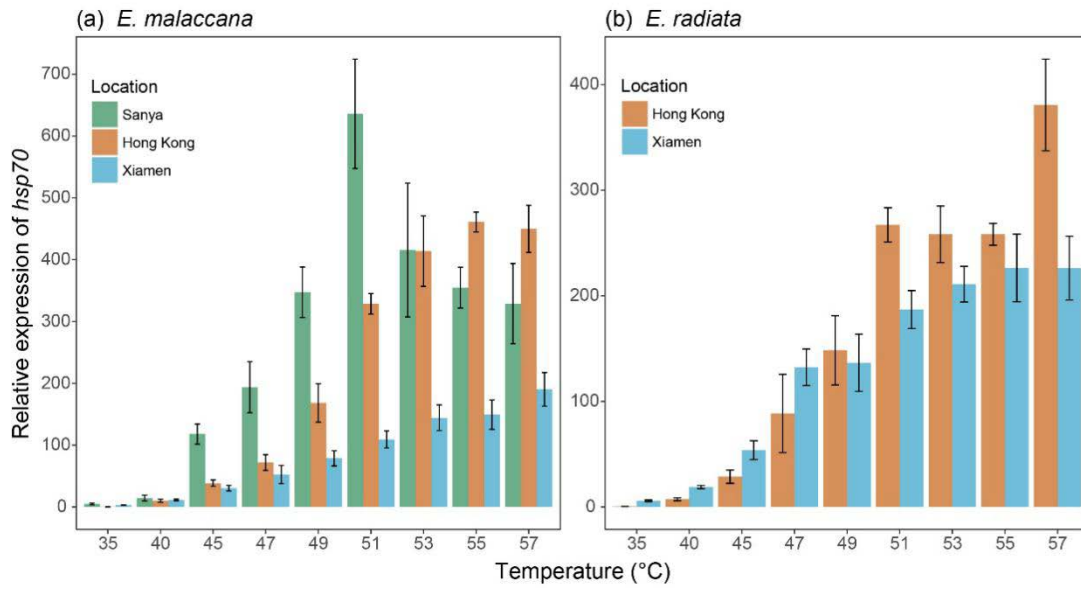


686

687 Figure 4. The mean *hsp70* levels of individuals collected from populations at the range  
 688 edge and range center (mean  $\pm$  SEM). \* indicates significant differences ( $P < 0.001$  for  
 689 both *E. malaccana* and *E. radiata*) between treatments (see text for further details).

690

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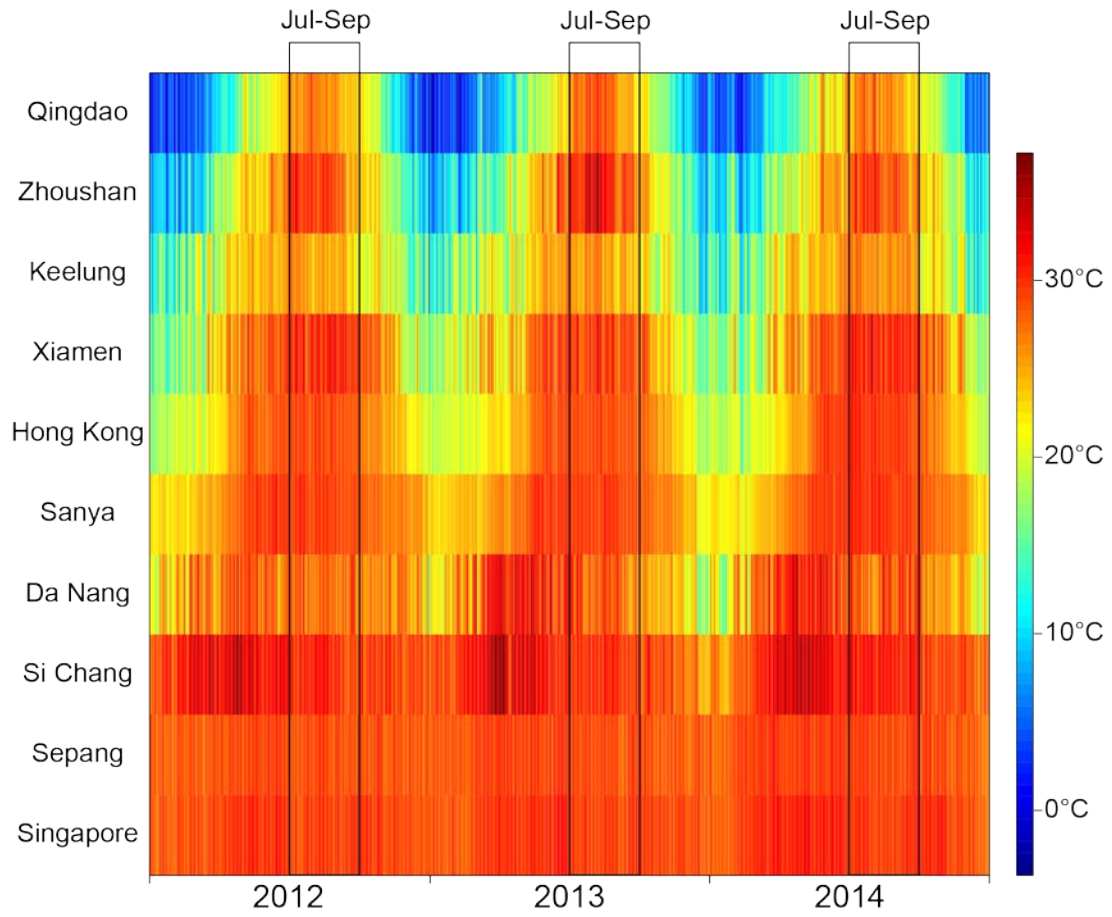


692

693 Figure 5. Relative expression of *hsp70* in response to experimental temperatures at 35, 40  
694 45, 47, 49, 51, 53, 55, 57°C (mean  $\pm$  SEM). Transcript patterns were shown for *E.*  
695 *malaccana* (a) and *E. radiata* (b) populations sampled in Xiamen, Hong and Sanya.

696

697



698

699 Figure S1. Color-coded charts showing daily maximal air temperatures for each sampling

700 location.

701