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The Role of Temperature and Desiccation Stress in Limiting the Local-scale Distribution of the Owl Limpet, *Lottia gigantea*

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2 **The role of temperature and desiccation stress in limiting the local-scale distribution of the**
3 **owl limpet, *Lottia gigantea***

4

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14

15 Running head: Stress and distributions of *Lottia gigantea*

16 Summary

- 17 1. Small scale distributions of rocky intertidal organisms may be determined in part by
18 temperature and desiccation stress during low tide and the ability of organisms to resist
19 such stresses. Biophysical modeling techniques, coupled with data on the physiological
20 tolerance of organisms can be used to predict the frequency and severity of abiotic stress
21 events.
- 22 2. The limpet *Lottia gigantea* is a major competitor for primary space on rocky shores
23 along the west coast of North America. The goal of this study was to quantify the thermal
24 and desiccation tolerance of this species and to predict the frequency of stressful events at
25 Hopkins Marine Station (HMS) in Pacific Grove, California, USA.
- 26 3. *L. gigantea* were exposed to thermal stress regimes in the laboratory using protocols
27 designed to mimic prolonged exposures in the field. The production of heat shock protein
28 70 (Hsp70) was measured as an indicator of sublethal stress, and mortality rates were
29 measured.
- 30 4. Peak expression of Hsp70 occurred during exposures at 32°C, while the lethal limits of
31 the limpets ranged between 30°C and 42°C, depending on the exposure regime. Lethal
32 temperatures were approximately 5°C lower in 50-60% relative humidity conditions
33 compared to 100% relative humidity trials.
- 34 5. Results from the physiology experiments were compared to field surveys of the upper
35 limits of *L. gigantea* at HMS and with heat-budget model predictions of limpet body
36 temperatures on a variety of substratum orientations.
- 37 6. The highest frequency of sublethal and lethal stress events was predicted to occur on
38 high-shore westerly and southerly slopes and horizontal surfaces, microhabitats where *L.*

39 *gigantea* is comparatively rare at HMS. Predicted stress and mortality events were more
40 rare at low-shore sites, vertical surfaces, and sloping rock faces oriented towards the
41 northeast and northwest.

42 7. The combination of direct measurements of sublethal and lethal temperature thresholds
43 using realistic stress exposures and predictions from a heat-budget model give insight
44 into the role of thermal disturbance in affecting the local-scale distributions of intertidal
45 organisms. These methods hold great promise for enhancing our understanding of the
46 role of abiotic factors in limiting species distributions and making predictions about
47 responses to future climatic conditions.

48

49 Keywords: heat budget model, rocky intertidal zone, heat shock protein 70, body temperature,
50 shore height, solar irradiance, lethal temperature, wave exposure

51 INTRODUCTION

52 In both the terrestrial and marine realms, species distributions can be influenced by
53 abiotic factors, including extremes in environmental conditions such as temperature,
54 precipitation, desiccation, wind and wave forces. On mountain tops, small excursions along the
55 vertical and horizontal axes can produce marked changes in wind and sun exposure, snow
56 accumulation, precipitation and temperature, changes that are often reflected in the biological
57 community (Poore & McVean 1957, Johnson & Billings 1962, Ferreyra et al. 1998). In the
58 intertidal zone, abiotic conditions may shift even more dramatically over scales of just a few
59 meters. For organisms that live at this interface of the marine and terrestrial realms, survival
60 requires withstanding a broad array of abiotic and biotic stresses.

61 The distribution of a species on the shore is set by numerous factors, such as interactions
62 with other species, that occur within a constantly shifting set of environmental parameters as the
63 tide rises and falls (Stephenson & Stephenson 1949, Southward 1958, Connell 1961, Newell
64 1964). The potential for high temperatures and desiccation to limit the range of an organism can
65 be intimately tied to the small-scale variation in conditions that exists between fully submerged
66 and fully terrestrial habitats, along with the physiological tolerance of a species to conditions in
67 each microhabitat (Helmuth & Hofmann 2001, Somero 2002, Davenport & Davenport 2005,
68 Bertness et al. 2006). The role of thermal and desiccation stress in setting the distributional limits
69 of organisms, over both large and small scales, has been an integral part of studies of this system
70 (Broekhuysen 1940, Lewis 1954, Foster 1971, Wolcott 1973, Branch 1981, Williams & Morritt
71 1995, Stillman & Somero 2000, Harley 2003, Harley & Helmuth 2003, Harley 2008).

72 On a rocky shore, there are three principal factors that determine the suitability of a
73 habitat for occupation with regard to temperature and desiccation stress. First, height on the

74 shore determines the total amount of time the organism is exposed to the air, a time during which
75 temperature may fluctuate substantially (Helmuth 1999, Denny, Miller & Harley 2006, Finke,
76 Navarrete & Bozinovic 2007). Second, the orientation of the substratum and neighboring
77 structures (including other species) affects the amount of solar radiation received by an
78 organism, and thereby its heat influx (Gates 1980, Campbell & Norman 1998). Finally, the
79 orientation of the microhabitat relative to incoming waves, coupled with local topographic
80 features which can increase or decrease wave swash, influences the degree of temperature
81 buffering provided by seawater (Harley & Helmuth 2003, Helmuth & Denny 2003, O'Donnell &
82 Denny 2008).

83 These environmental characteristics of a microhabitat can be used to predict the potential
84 severity of temperature and desiccation stress of that site. A biophysical model of body
85 temperature, generally referred to as a heat-budget model, allows researchers to make hindcasts
86 or forecasts of an organism's body temperature, using a variety of environmental data and
87 selected physical characteristics of the organism (Porter & Gates 1969, Bell 1995, Helmuth
88 1999, Denny & Harley 2006). With a heat-budget model in hand, one can begin to speak to the
89 suitability of a particular microhabitat, with regard to temperature and desiccation stress, for the
90 organism (Denny et al. 2006). To make quantitative predictions about the effect of stress on the
91 distributions of the organism, however, this process requires knowledge of the physiological
92 tolerance of the organism (Newell 1976, Branch 1981, Davenport & Davenport 2005, Helmuth et
93 al. 2006).

94 The focus of this study is the owl limpet *Lottia gigantea* Sowerby, 1834, a common
95 competitor for primary space in the mid-intertidal zone on rocky shores from central California
96 to northern Baja California (Stimson 1973). These limpets graze territories on the rock surface,

97 feeding on algae and actively removing other organisms that intrude on the territory (Lindberg,
98 Estes & Warheit 1998). Individual adult *L. gigantea* typically occupy a territory for multiple
99 years and may be restricted to that area by the actions of neighboring limpets or other species
100 occupying primary space around the territory (Galbraith 1965, Stimson 1970). *L. gigantea* is
101 typically found on wave-exposed shores on vertical or sloped substratum, occasionally occurring
102 on horizontal platforms (Denny & Blanchette 2000). We detail a combination of physiological
103 and biophysical techniques, along with field surveys of limpet upper limits, aimed at testing the
104 hypothesis that the upper limits and along-shore distribution of *L. gigantea* could be driven, in
105 part, by physiological limitations in the response to extreme temperatures and desiccation stress
106 during aerial emersion. Can the upper vertical limits of *L. gigantea* populations be set by
107 occasional high temperature events during low tide periods, or are they physiologically robust
108 enough that rare, stressful low tides are inconsequential? By measuring the temperature and
109 desiccation tolerance of *L. gigantea* in conditions that approximate stressful events from the
110 field, and comparing these data with hindcasts of limpet body temperatures produced by a heat-
111 budget model in various microhabitats, we predict which microhabitats should be unsuitable for
112 long-term occupation by *L. gigantea*. These predictions are then compared to observed
113 distributional patterns in the field.

114 MATERIALS AND METHODS

115 *Collections*

116 All *Lottia gigantea* were collected from north and northeast facing vertical or near-
117 vertical walls in the intertidal zone at Hopkins Marine Station (HMS), Pacific Grove, California
118 (36° 37.3' N, 121° 54.25' W). Collections were made between December 2006 and March 2007.

119 Prior to all experiments, limpets were held in an outdoor seawater table for a minimum of five
120 days, where they were splashed with seawater and were able to graze freely. The water
121 temperature in the holding table was the same as the ambient seawater temperature off HMS,
122 typically between 11 and 14°C. The outdoor aquaria were shaded from the sun, and air
123 temperatures ranged between approximately 8 and 20°C.

124 *Laboratory heat stress experiments*

125 To produce heat stress profiles similar to those experienced in the field by limpets, a
126 temperature-controlled recirculating wind tunnel allowed the body temperature of limpets to be
127 raised gradually by manipulating the temperature of the substratum and air. Using output from
128 the heat-budget model developed by Denny and Harley (2006) for *L. gigantea*, a heating rate of
129 8°C hr⁻¹ was chosen to represent typical conditions during an extreme high temperature event in
130 the field. The relative humidity in the chamber was maintained between 50 and 60%, based on
131 relative humidity measurements taken next to *L. gigantea* in the field during prolonged heat
132 stress events (L. Miller, pers. obs.). In the field, limpet body temperature is largely dependent on
133 substratum temperature due to the large contact area of the foot (Denny & Harley 2006). In the
134 wind tunnel, limpets sat on a heated aluminum plate that was used to control limpet body
135 temperature while air was circulated through the tunnel at 0.5 m s⁻¹. Each heat stress trial ran for
136 a total of 7 h, with the temperature starting and ending at 14°C, and changing at the fixed 8°C h⁻¹
137 rate. When limpets reached the designated maximum body temperature in the middle of a trial,
138 that temperature was held steady until it was time to begin reducing the temperature to 14°C
139 (again at 8 °C h⁻¹) all within the 7 h time limit (Fig. 1). Air temperature in the wind tunnel was
140 raised and lowered at 8 °C h⁻¹ from the same 14°C starting point. Air temperature in the field at

141 HMS rarely exceeds 30°C, even on the warmest days, so all wind tunnel experiments used a
142 maximum air temperature 30°C.

143 The recirculating wind tunnel was used to quantify survival of *L. gigantea* at high
144 temperatures. Each lethal temperature trial used 5 *L. gigantea*, and each temperature trial was
145 replicated three times. Maximum body temperatures in these trials were 30, 32, 34, 36 or 38°C.
146 Following a heat stress exposure, experimental limpets were returned to running seawater for 24
147 h. If the limpet could not maintain a grip on the substratum after 24 h, it was scored as
148 “ecologically dead” (Wolcott 1973).

149 Intertidal organisms’ survival at high temperatures has often been assayed while the
150 organisms were either submerged or emersed at high humidity (Sanders *et al.* 1991, Clarke, Mill
151 & Grahame 2000, McMahon 2001, Somero 2002, Davenport & Davenport 2005). Unlike the
152 experiments described above, these procedures precluded desiccation of the test limpets during
153 heat stress. To facilitate comparison between these methodologies and the more realistic wind-
154 tunnel experiments described above, the thermal tolerance of *L. gigantea* was also determined
155 while emersed at 95-100% relative humidity. A simple closed chamber was designed with a
156 platform to hold the limpets in air above a reserve of seawater in the lower section. The
157 temperature inside the chamber was raised and lowered using the same temperature profiles used
158 in the wind tunnel, and survival was assayed as described above. Trials were run using maximum
159 temperatures of 32, 34, 36, 38, 40 and 42°C. Groups of five limpets were used in each
160 temperature trial, and each trial was replicated three times.

161 The number of surviving limpets in each trial was expressed as a fraction *S* of the total
162 number of limpets in the trial. *S* was averaged for the three replicates at each temperature. The

163 lethal temperature, T , at which 50% of the limpets in a trial died (LT50) was estimated by a
164 fitting a sigmoidal curve to the survival data:

$$S = 1 - \frac{1}{1 + \exp\left(-\frac{T - a}{b}\right)} \quad (1)$$

165 where a and b are best-fit coefficients determined by a curve-fitting routine implemented in
166 Matlab software (The Mathworks, Natick, MA, USA). The value of a is equal to the LT50
167 temperature.

168 *Sublethal stress*

169 In addition to measuring lethal limits for *L. gigantea*, the recirculating wind tunnel was
170 used to expose limpets to sub-lethal temperature and desiccation stress. The intensity of sub-
171 lethal stress was measured using a common physiological stress marker, heat shock protein 70
172 (Hsp70). Groups of five *L. gigantea* were stressed in the recirculating wind tunnel using
173 maximum temperatures ranging from 24 to 36°C for 7 h using the same exposure profiles as the
174 lethal temperature determinations. To control for the effects of handling and exposure in the
175 environmental chamber, a control group of five limpets was placed in the environmental
176 chamber and held at 14°C for 7 h. These control limpets remained wetted for the entire duration
177 of the exposure, so that desiccation was likely a minor stress. The limpets targeted for protein
178 expression work recovered in flowing seawater for one hour, and were then frozen in liquid
179 nitrogen. An additional field control sample of five *L. gigantea* was collected from the field at
180 HMS while still submerged by a high tide. These limpets were immediately frozen following
181 collection. The frozen limpets were stored at -70°C until they could be processed.

182 Hsp70 assays were carried out using a protocol similar to that of Hofmann and Somero
183 (1995). Each limpet was partially thawed and a small portion of the pallial gills and mantle tissue
184 (~1 – 2 mg) was dissected from the edge of the mantle (Fisher 1904). Tissues were homogenized
185 in buffer by boiling for 5 min followed by 2 min. in an automatic homogenizer (Tissuelyser,
186 Qiagen, Valencia, CA, USA). The samples were boiled and homogenized twice more before
187 centrifugation at 14,000 g for 15 min. The supernatant was pipetted to a new microfuge tube and
188 stored at -20°C.

189 Quantification of the total protein content of each sample was carried out using the BCA
190 Pierce protocol (BCA Protein Assay Kit, Pierce Inc.) in 200 µl well plates. Each sample was
191 loaded in triplicate, and each plate contained a set of pre-diluted bovine serum albumin (BSA)
192 standards, also loaded in triplicate. Absorbances were measured in a SpectraMax 340pc
193 spectrophotometer (Molecular Devices, Sunnyvale, California, USA). A standard curve was
194 calculated from the absorbance of the standards, and the average of the three replicates of each
195 sample was taken as the protein concentration for that sample. The protein quantification was
196 carried out two times in order to determine if concentrations changed appreciably due to freeze
197 and thaw cycles, but no change was detected.

198 SDS-PAGE gel electrophoresis was used to separate proteins for western blotting. After
199 boiling for 3 minutes at 100°C and centrifuging at 14,000 g for one minute, 7 µg of total protein
200 from each limpet, along with Laemli sample buffer and distilled water, were loaded into a lane of
201 a 10% Tris-HCl pre-cast gel (Ready-Gel Polyacrylamide gel, Bio-Rad Laboratories, Hercules,
202 CA, USA). In addition to the heat-shocked samples, a molecular weight standard was loaded
203 (Precision Plus Protein Standard, Bio-Rad Laboratories, Hercules, CA, USA), along with 30 ng
204 of human Hsp70 in another lane (NSP-555, StressGen – Assay Designs, Ann Arbor, MI, USA).

205 Gels were submerged in Tris-glycine running buffer with 0.1% SDS. The gels were placed in a
206 4°C cold room and electrophoresed at 200 V for 50 min.

207 The proteins were then electrophoretically transferred from the gel to the solid phase on
208 nitrocellulose membranes. Each gel was sandwiched with the nitrocellulose membrane between
209 four pieces of filter paper and submerged in transfer buffer (Tris-glycine and 20% methanol).
210 The transfer was carried out in the 4°C cold room at 80 V for 75 min.

211 Membranes were stored dry before the start of the immunoassay. Each membrane was
212 incubated with gentle shaking in blocking buffer (phosphate-buffered saline (PBS) with 5% non-
213 fat dried milk and 0.1% Tween-20) for 1 h. After three 5 min washes in PBS, membranes were
214 incubated for 1 hour with gentle shaking in primary antibody mixed 1:5000 in PBS with 5%
215 BSA (antibody MA3-008, clone 2A4, mouse monoclonal, Affinity BioReagents). The
216 membranes were washed six more times for 5 min each prior to incubation in the secondary
217 antibody (SAB-100, 1:5000 dilution in PBS with 5% BSA, StressGen – Assay Designs). The
218 incubation in secondary antibody (1.5 h), was followed by three, 5 min washes in PBS with 0.1%
219 Tween-20. Proteins were visualized by exposing the membranes to enhanced chemiluminescence
220 reagents (GE Healthcare Bio-Sciences Corp) and exposing to x-ray film. Multiple exposure
221 times between 30 s and 20 min. were used for each membrane to ensure proper exposure.

222 The exposed x-ray film was digitally scanned on a transparency scanner. The image files
223 were transferred to the image analysis program Image-J (Rasband 1997-2009) for densitometry.
224 The density of each sample band at 70 kDa was measured relative to the density of the 30 ng
225 human Hsp70 standard on each gel. The presence of the human standard on all gels allowed for
226 comparison of relative density values across multiple western blots. Data were log transformed

227 to better conform to assumptions of normality and tested for homogeneity of variances using
228 Cochran's test. The results for the limpets collected at high tide in the field were not included in
229 the statistical analysis due to the difference in protocol, since they were not acclimated in the lab
230 or handled during experiments.

231 *Limpet upper limits*

232 We surveyed the upper limits of *L. gigantea* at HMS during October 2008. Between five
233 and seven sites facing northwest, northeast, southwest or southeast were surveyed. Much of the
234 intertidal substrate at HMS faces in one of these four directions. Each site was comprised of a
235 smooth rock face sloped between 30° and 70° above horizontal. The height of the highest *L.*
236 *gigantea* on each face was measured using a theodolite and stadia rod with reference to a U.S.
237 National Geodetic Survey marker located at HMS. These heights were then converted to height
238 above MLLW (National Tidal Datum Epoch 1983-2001, Monterey, CA).

239 *Heat-budget modeling*

240 The heat-budget model of Denny and Harley (2006) was used to produce hindcasts of *L.*
241 *gigantea* body temperatures on a number of hypothetical shore positions at HMS. The model,
242 written and run using Matlab software, used the same parameters as the previously published
243 model. An expanded 7 year set of historical weather data collected at HMS, from 1 August 1999
244 through 31 July 2006, was used to produce a time series of body temperatures at 10 min intervals
245 for the seven year period. The modeled limpet was 42 mm long, and was positioned in one of 5
246 orientations: on a horizontal surface, or on sloped surfaces elevated above horizontal in 10°
247 increments, facing different directions (north, east, south etc) around the compass in 22.5° steps.
248 The limpets were modeled at elevations of 0.5 m to 2.0 m above Mean Lower Low Water

249 (MLLW) in 0.5 meter increments on a simulated wave-protected shore (i.e., no wave splash).
250 These conditions were meant to produce a “worst-case scenario” for *L. gigantea* on the shore at
251 HMS.

252 The predictions from the heat-budget model were used to calculate the frequency and
253 severity of high temperature stress events over a 7 year period at HMS. Additionally, the
254 conditions on days leading up to stress events were examined to address the potential for short-
255 term physiological acclimation to high temperatures. We identified 130 days in the 7 year
256 temperature record where the body temperature of a limpet on a southwest-facing sloped rock at
257 2.0 m above MLLW was predicted to exceed 28°C. For each day predicted to raise body
258 temperatures above this threshold, the temperature profiles from the previous two weeks were
259 extracted from the data. The maximum temperature on each of the 14 days prior to each high
260 temperature event was sampled, and the mean difference between the daily maximum body
261 temperature and the 28°C threshold was calculated for each day.

262 RESULTS

263 *Lethal temperature trials*

264 When *L. gigantea* were subjected to a 7 h aerial exposure regime at 100% relative
265 humidity, the median lethal temperature (LT50) was 37.4°C (Fig. 2). Limpets died at maximum
266 temperatures as low as 34°C, and all limpets died when the maximum temperature was 42°C.

267 Survival rates in the recirculating wind tunnel at a more realistic 50-60% relative
268 humidity were lower than the high-humidity treatments (Fig. 2). The LT50 for limpets in the
269 low-humidity trials was 32.5°C, with mortality beginning to occur at 30°C.

270 *Sublethal temperature trials*

271 The primary antibody used in these experiments resolved one band of 70 kDa that
272 showed low levels of constitutive expression and was strongly induced by temperature stress
273 (Fig. 3a). There was a significant effect of temperature treatment on Hsp70 expression (ANOVA,
274 $F_{4,20} = 4.66, P = 0.008$) for limpets stressed in the recirculating wind tunnel. The mean density of
275 the Hsp70 band increased in treatments with increasing temperature up to 32°C, beyond which
276 expression declined (Fig. 3b). Field control limpets exhibited the lowest levels of inducible
277 Hsp70 expression.

278 *Limpet upper limits*

279 The upper limit of *L. gigantea* differed significantly among the four shore directions
280 surveyed (Kruskal-Wallis test, $X^2_{0.05,3} = 17.94, p < 0.001$). Sites facing northwest and northeast
281 had significantly higher upper limits than southwest facing sites (post-hoc comparison, Zar
282 1999), with southeast sites falling in between the two groups (Fig. 4).

283 *Heat-budget model*

284 Using the results from the sublethal and lethal stress experiments, two threshold
285 conditions were chosen to define “stressful” conditions in the heat-budget model. The threshold
286 for the onset of Hsp70 expression was defined as any day where the limpet body temperature in
287 the model was predicted to exceed 28°C for more than 3.5 h. This corresponded to the minimal
288 exposure regime in the recirculating wind tunnel that produced a significant increase in Hsp70
289 relative to control conditions. The mortality threshold used in the heat-budget model was defined
290 as any day where limpet body temperature was predicted to exceed 32°C for more than 2.5 h,
291 based on the lowest temperature conditions conservatively expected to produce mortality in the

292 recirculating wind tunnel trials. The cumulative number of stress events varied with the altitude,
293 azimuth, and shore height of the modeled substratum (Fig. 5), with southwest-facing sites angled
294 between 40 and 50° above horizontal producing the largest number of high temperature days,
295 and higher shore heights producing higher peak temperatures. The greatest number of mortality
296 events, six events in 6 years, occurred at a south-southwest facing slope elevated 50° above
297 horizontal at 2.0 m above MLLW. Results are shown for 1.0, 1.5 and 2.0 m above MLLW (Fig.
298 5), while the number of sublethal stress and mortality events at 0.5 m above MLLW approached
299 zero.

300 A representative set of temperature traces for one the most stressful modeled orientations,
301 a southwest-facing slope oriented 40° above horizontal at 2.0 m above MLLW, are shown in Fig.
302 6. The threshold for onset of Hsp70 production was exceeded 46 times at this model site, with
303 many of these exposures lasting more than the 3.5 h time threshold of our wind-tunnel
304 experiments. These prolonged exposures should further increase the thermal and desiccation
305 stress on the limpet above that measured here, resulting in more thermally-induced protein
306 damage and subsequent Hsp70 production. Over the 7 years of weather data used in the model,
307 only 6 days were predicted to exceed the mortality threshold of *L. gigantea* on the southwest-
308 facing slope, but those included 2 days with exposures sufficient to kill 70-90% of the limpets at
309 the modeled sites (i.e. body temperatures in excess of 34°C for more than 2 h).

310 An analysis of the conditions during the 2 weeks preceding a day where body
311 temperature was predicted to exceed 28°C for a limpet on a southwest slope at 2.0 m above
312 MLLW is shown in Fig. 7a. The mean difference between the maximum temperature on each
313 day leading up to the stress event and a 28°C threshold (\pm 95% confidence limits) was
314 calculated. Average daily maximum temperatures were on average 2.6°C cooler than the 28°C

315 threshold during the 2 weeks prior to a high temperature event, but this difference decreased as
316 the target date drew closer. The 1 to 2 days preceding a day where limpet body temperature
317 exceeded 28°C often consisted of maximum body temperatures that equaled or exceeded the
318 temperature on the target day, indicating that many stress events could be multi-day occurrences
319 rather than isolated events. We must include the caveat that the mean differences and confidence
320 limits were calculated as if each day leading up to a high temperature event was an independent
321 event, but there may be some autocorrelation among conditions on adjacent days. Two
322 representative periods leading up to hot days are shown in Fig. 7b-c. Conditions preceding a
323 28°C day may be consistently mild (Fig. 7b) or may consist of repeated days where body
324 temperature reaches or exceeds the 28°C threshold (Fig. 7c)

325 DISCUSSION

326 *Thermal stress and limpet distributions*

327 The rocky intertidal zone is commonly acknowledged as one of the most stressful habitats
328 on earth. The steep gradients in conditions along both horizontal and vertical axes can create
329 greatly different conditions within microhabitats separated by only 10's of centimeters. For
330 mobile organisms, or during the initial settlement period for sessile organisms, the choice of
331 microhabitat can mitigate or intensify the severity of abiotic stresses, influencing survival and
332 fitness (Williams & Morritt 1995, Davison & Pearson 1996, Bertness et al. 1999, Gray &
333 Hodgson 2004).

334 For *L. gigantea*, upper distributional limits on vertical walls or sloped northeast-facing
335 microhabitats at HMS should not be limited by thermal stress anywhere in the 0.5 to 2.0 m
336 vertical zone modeled here. The *L. gigantea* used in the wind tunnel experiments survived

337 temperature and desiccation stress levels well beyond the most stressful conditions predicted by
338 the heat-budget model for these microhabitats during the study period. Northwest-facing slopes
339 were also generally benign, with steeply sloped habitats producing 0 – 10 sublethal-stress events
340 and no mortality events at upper shore levels during the seven year timeseries.

341 In contrast, high-shore conditions at HMS at horizontal or southwest- and southeast-facing
342 sloped microhabitats could be severe enough to limit the vertical distribution of *L. gigantea*.
343 These sites receive more solar irradiance, particularly during the middle of the day when the sun
344 is high overhead and air temperatures are highest (Campbell & Norman 1998). The resulting
345 predicted limpet body temperatures exceeded the mortality threshold determined in the
346 laboratory between two and six times during the 7 years of model data, depending on site
347 orientation and height. In earlier work (Denny et al. 2006), we concluded that acute thermal
348 mortality was not sufficient to set an upper limit to the vertical range of *L. gigantea*. The longer
349 stress exposure used here, 7 h vs. 3.5 h, lowered the mortality temperature threshold sufficiently
350 to predict occasional acute temperature-induced mortality at higher shore levels on these sun-
351 exposed surfaces. From our field surveys, the general pattern of height of the upper limit of *L.*
352 *gigantea* progresses NE>NW>SE>SW, which corresponds to the predicted incidence of stressful
353 conditions at these microhabitat types produced by the heat-budget model.

354 Occasional acute temperature and desiccation stresses have been reported to cause
355 mortality and potentially alter the upper limits of intertidal species (Sutherland 1970, Foster
356 1971, Tsuchiya 1983, Wethey 1983, Bertness et al. 1999, Harley 2008). The study of these
357 events is often complicated by their rarity, driven by the need for a confluence of ocean and
358 weather conditions sufficient to raise temperatures to extreme levels (Denny et al *in press*). Calm
359 seas, a mid-day low tide, and warm, sunny conditions must occur simultaneously. At HMS, these

360 conditions occur only a few times per year, and the frequency of potentially lethal high
361 temperatures is therefore very low within a given year. However, *L. gigantea* is a long-lived
362 species, with individuals living for more than 20 years (Fenberg 2008), which increases the
363 chances of experiencing these rare stress events. Our observations of *L. gigantea* distributions at
364 HMS agree with the predictions of the heat-budget model and physiology experiments. *L.*
365 *gigantea* that live on open rock faces tend to cover a larger vertical range on northwest- and
366 northeast-facing slopes compared to southwest and southeast-facing slopes. However, these
367 patterns in the upper limit of *L. gigantea* cannot be solely attributed to the influence of acute
368 thermal stress events. The intertidal zone at HMS is primarily oriented northward and eastward,
369 with ocean swells approaching from the north, so that the total area available for habitation
370 should be greater on rocks of these aspects. The high relief of the granite topography also
371 provides many microhabitats that could serve as refuges from thermal stress by shading
372 organisms and concentrating wave splash (O'Donnell & Denny 2008), allowing *L. gigantea* to
373 extend their territories further up the shore.

374 The upper limits of limpet distributions can also be potentially set by other abiotic and
375 biotic factors. Both competitive and facilitative interactions with neighboring species can
376 influence the suitability of a patch of rock. Other grazers may reduce the standing crop of algae
377 (Castenholz 1961), while sessile organisms such as macroalgae, barnacles and mussels may
378 occupy potential limpet territories (Stimson 1970, 1973). Alternatively, neighboring algae and
379 mussels could provide beneficial shade and retain moisture, ameliorating temperature and
380 desiccation stresses at small scales (Bertness & Leonard 1997, Bertness et al. 1999). At higher
381 shore heights, the time of emersion and higher solar irradiation may limit the growth of the

382 microalgal food supply of *L. gigantea*, making the habitat unsuitable for occupation (Castenholz
383 1961).

384 Beyond rare mortality events, the frequency and severity of sublethal stress events could
385 limit the distribution of *L. gigantea* as well. The expression of Hsp70, a common marker for
386 general physiological stress (Feder & Hofmann 1999), was induced between 24 and 28°C in *L.*
387 *gigantea*. It should be noted that the recovery time used in these experiments (1 h) was relatively
388 short, and longer recovery times may have revealed enhanced Hsp expression at lower
389 temperatures (Hofmann & Somero 1996; Tomanek & Somero 2000). Based on these data,
390 sublethal stress events could occur multiple times per year in many of the microhabitats at HMS.
391 Expression of Hsp's carries great metabolic costs as well as indicating that the organism has
392 sustained some measure of cellular damage (Hofmann & Somero 1996, Feder & Hofmann 1999,
393 Tomanek 2002). We are only beginning to understand the long-term effects of repeated sublethal
394 stress events on the survival, growth, and reproduction of intertidal organisms (Helmuth &
395 Hofmann 2001, Somero 2002). The range of onset temperatures for Hsp70 production in *L.*
396 *gigantea* resembles that of other mid-intertidal species from the west coast of North America
397 including other limpets, snails, and mussels (Roberts, Hofmann & Somero 1997, Tomanek &
398 Sanford 2003, Dong et al. 2008). The environmental conditions on horizontal platforms or on
399 southwest- and southeast-facing sloped rocks could be severe enough that repeated sublethal
400 temperature and desiccation stresses inhibit the fitness of *L. gigantea* living there, but this
401 remains unstudied.

402 Care must be taken in any generalization of our predictions from the heat-budget model.
403 The frequency, intensity, and duration of thermal and desiccation stress events in the intertidal
404 zone are intimately associated with the particular microhabitat of an organism (Grainger 1969,

405 Denny et al. 2006). Organisms that seek refuge in crevices, under an algal canopy, or in the
406 interstices of biogenic structures such as mussel beds will experience fewer stressful days due to
407 shading and retention of moisture (Kensler 1967, Raffaelli & Hughes 1978, Halpin et al. 2002,
408 Gray & Hodgson 2004, Bazterrica et al. 2007). Organisms that live out on open surfaces should
409 be in much greater danger of reaching extreme temperatures (Breen 1972, Roberts, Hofmann &
410 Somero 1997, Gilman 2006, Berger & Emler 2007, Jost & Helmuth 2007). *L. gigantea*
411 represents a good model organism for this type of modeling, as the territorial nature of this
412 species often restricts it to a defined area on the substratum that has little or no canopy cover. For
413 limpets thus constrained, the model does a good job of predicting body temperature, especially
414 on warm days (Denny & Harley 2006).

415 *Applying laboratory data to the field*

416 Accurately predicting how intertidal organisms will cope with prolonged aerial exposure
417 during low tide requires experimental methods that reasonably recreate conditions in the field
418 (Newell 1976, Branch 1981, Helmuth et al. 2006, Jost & Helmuth 2007). Simplified laboratory
419 stress protocols have traditionally been used for comparative physiological studies, creating
420 difficulties in the transition from idealized conditions in the laboratory to the extremely variable
421 conditions of the real world. These difficulties raise a number of concerns that must be addressed
422 before we can produce meaningful measures of thermal and desiccation tolerance for intertidal
423 species. Much of the existing literature on thermal stress in intertidal organisms relies on lethal
424 or sublethal experimental data derived from high humidity trials in closed chambers or tests in
425 which organisms were submerged in seawater (Broekhuysen 1940, Orr 1955, Fraenkel 1968,
426 Stirling 1982, McMahon & Britton 1985, Cleland & McMahon 1986, McMahon 1991, Sanders
427 et al. 1991, Tomanek & Somero 1999, Clarke, Mill & Grahame 2000, Tomanek & Somero 2000,

428 McMahon 2001, Sorte & Hofmann 2005, Davenport & Davenport 2005, Berger & Emlet 2007).

429 The prevalence of these protocols is understandable from a logistical standpoint, as the high-

430 humidity or submerged trials can be carried out with simple water baths. Because much of the

431 work on thermal tolerance in intertidal organisms has been focused on comparisons across

432 species, these standardized methods allow researchers to remove much of the variability of the

433 natural system. However, these methods have the potential to be misleading when the goal is to

434 predict performance in the field. In the current study, *L. gigantea* were able to survive much

435 higher temperatures when kept in saturated air (Fig. 2), a condition that is extremely unlikely to

436 occur in the field at HMS. The median lethal temperatures for the 100% and 50% relative

437 humidity trials decreased by nearly 5°C in the lower humidity trial. Clearly the method by which

438 lethal temperature determinations are made will have substantial effects on the application of the

439 data to species performance in the field. The practice of exposing intertidal organisms to thermal

440 stress in seawater could complicate comparisons with both 100% RH and low-humidity aerial

441 exposures due a number of factors. Submerged respiration rates and aerial respiration rates may

442 differ markedly (Carefoot 1987). In air, the inability to dispose of metabolic waste products can

443 create changes in the osmolality of both internal and external water stores, while desiccation will

444 further concentrate solutes in the remaining water at low humidity, enhancing physiological

445 stress (Segal & Dehnel 1962, Wolcott 1973, Newell 1976, Lowell 1984). For these reasons,

446 caution is warranted when trying to apply results from submerged stress trials to emersion in the

447 field, as they may overestimate the tolerance of the organism to stressful emersed conditions.

448 Although the lethal temperatures differed greatly between the treatments used here, the onset

449 temperature for the heat shock response of *L. gigantea* (24 - 28°C) was similar to previously

450 published values for related limpets stressed in water (23 – 29°C, Sanders et al 1991) and for

451 *Chlorostoma (Tegula)* snails stressed in water (24-27°C, Tomanek and Somero 1999) or at low
452 tide in the field (24-27°C, Tomanek and Sanford 2003).

453 The time and temperature profile of stress exposures used here represents only a small
454 fraction of the large variety of potential field conditions that a limpet might experience. The
455 maximum temperatures used in this study encompass the likely range of high temperatures that
456 occur in the field at HMS. However, based on the results from the heat-budget model, there is
457 wide latitude in the length of aerial emersion during low tide (Fig. 6). During calm ocean
458 conditions, *L. gigantea* living in the upper portion of their range at HMS might reasonably
459 remain emersed for 12 h or more, potentially causing even more severe physiological stress than
460 the conditions used in this study. In addition to the time of exposure, the rate of heating may
461 impact the extent of the heat shock response, potentially by damaging cellular processes before
462 the onset of heat shock protein production. The rate of heating has been demonstrated to affect
463 the thermal limits of insects (Chown et al 2009), but unfortunately studies on the effects of
464 heating rate in intertidal organisms are rare. Tomanek & Somero (2000) observed a pronounced
465 increase in the heat shock response of low-shore *Chlorostoma brunnea* when they were heated
466 relatively slowly in air compared to being immersed directly in hot water, but the related mid-
467 shore snail *Chlorostoma funebris* showed no difference in Hsp expression under the same
468 heating conditions. These results again demonstrate that comparisons between submerged and
469 emersed stress protocols should be approached with caution, as body temperature can respond
470 much more quickly to changes in water temperature than air temperature due to the thermal
471 conductivities of these fluids. Measuring the temperature of a water bath used to heat a container
472 full of air may give an inaccurate estimation of body temperature of the animal in the container,
473 so heating rate in air should be tied directly to the organism body temperature (as in the current

474 study). Model results from this study indicate that the rate of heating for *L. gigantea* in the field
475 may vary between approximately 2 and 10°C h⁻¹ on warm days (Fig. 6). Slower heating rates
476 must necessarily be accompanied by prolonged exposure times in order to reach stressful
477 temperatures, and the effects of this combination of conditions on *L. gigantea* are currently
478 unknown.

479 *Acclimatization*

480 Single, acute physiological stress events have been the focus of this study, but conditions
481 in the field may occasionally lead to repeated stress events over multiple days. The physiological
482 capacity of limpets to withstand repeated stressful days has not been explored in great detail.
483 Studies on other intertidal species have demonstrated that physiological acclimatization can
484 occur, altering the heat shock response and potentially shifting the lethal limits of an organism
485 (Markel 1974, Roberts et al. 1997, Tomanek & Somero 1999, Buckley, Owen & Hofmann 2001,
486 Tomanek 2005, but see Berger & Emler 2007). Physiological acclimatization may occur over
487 just a few days in response to weather conditions, and can happen on a seasonal time scale as
488 well (Newell 1976). Just as the lethal temperature for *L. gigantea* varies with the conditions
489 (time and humidity) in a single exposure, prior acclimatization to warmer seasonal temperatures
490 or repeated acute stress events will likely influence the survival of the limpet during a particular
491 stressful low tide.

492 Further study is required to determine the response of *L. gigantea* to these repeated multi-
493 day high temperature exposures. The results, in terms of sub-lethal stress or mortality, from
494 repeated events are difficult to predict. On the one hand, short-term physiological acclimatization
495 might occur and allow *L. gigantea* a better chance of surviving each subsequent exposure. On the

496 other hand, the cumulative effects of multi-day stress events could cause *L. gigantea* to succumb
497 to lower maximum temperatures.

498 *Conclusions*

499 Studies of the type described here represent progress towards the integration of
500 biomechanical and physiological techniques with the goal of testing ecological hypotheses (Huey
501 1991, Chown & Terblanche 2007). We can combine physiological performance data, gathered
502 under realistic conditions, with computer simulations of organismal temperatures in the field to
503 make predictions about where and when the distribution of an ecologically dominant species
504 may be limited by extreme temperature events. This study and others like it provide ecologists
505 with a robust set of tools to examine questions on the importance of thermal stress and
506 microhabitat selection in determining the identity of species that comprise the community at a
507 site. As with terrestrial species (Kearney & Porter 2004, Buckley & Roughgarden 2006,
508 Pincebourde et al 2007, Mitchell et al 2008, Kearney et al 2008), a growing number of
509 biophysical models for important intertidal species such as the limpets, mussels, and algae (Bell
510 1995, Helmuth 1999, Denny & Harley 2006, Gilman et al 2006), coupled with increasingly
511 detailed studies of the physiology of these organisms (Gracey et al 2008) will form the basis for
512 mechanistic models to study species interactions and make predictions about future community
513 development, especially in the face of an uncertain climatic future (Helmuth et al 2006).

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762 FIGURE LEGENDS

763 Fig. 1. Total elapsed time of all trials was 7 h.

764 Fig. 2. .

765 Fig. 3.

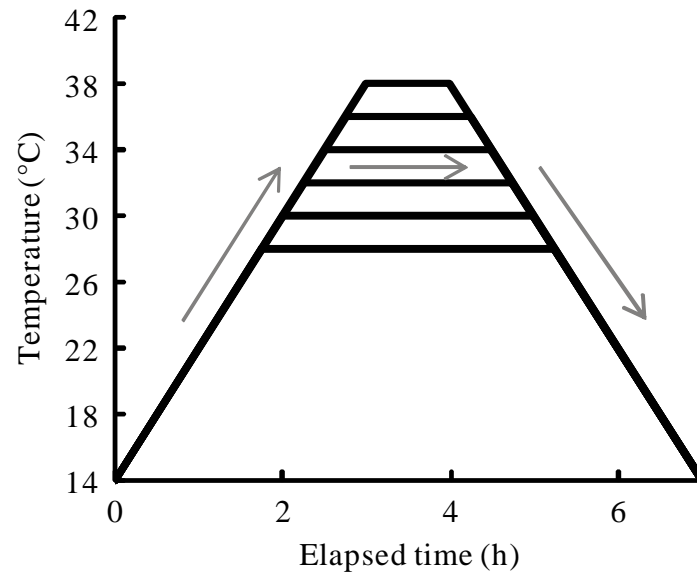
766 Fig. 4.

767 Fig. 5.

768 Fig. 6.

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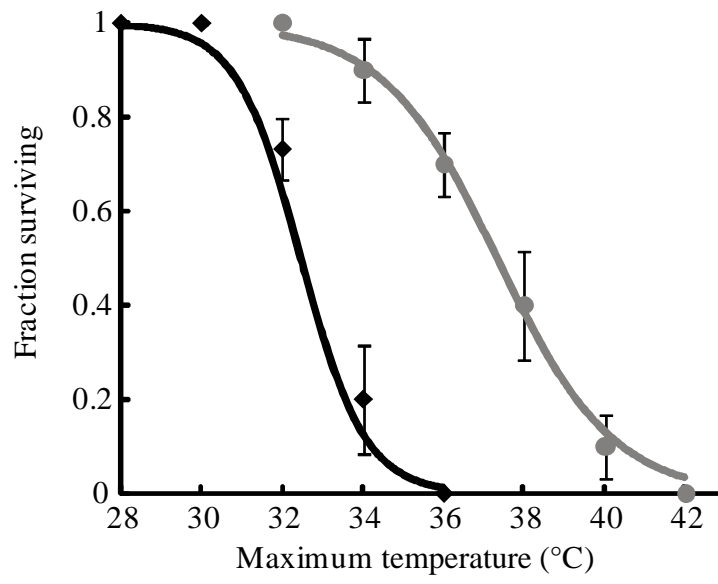
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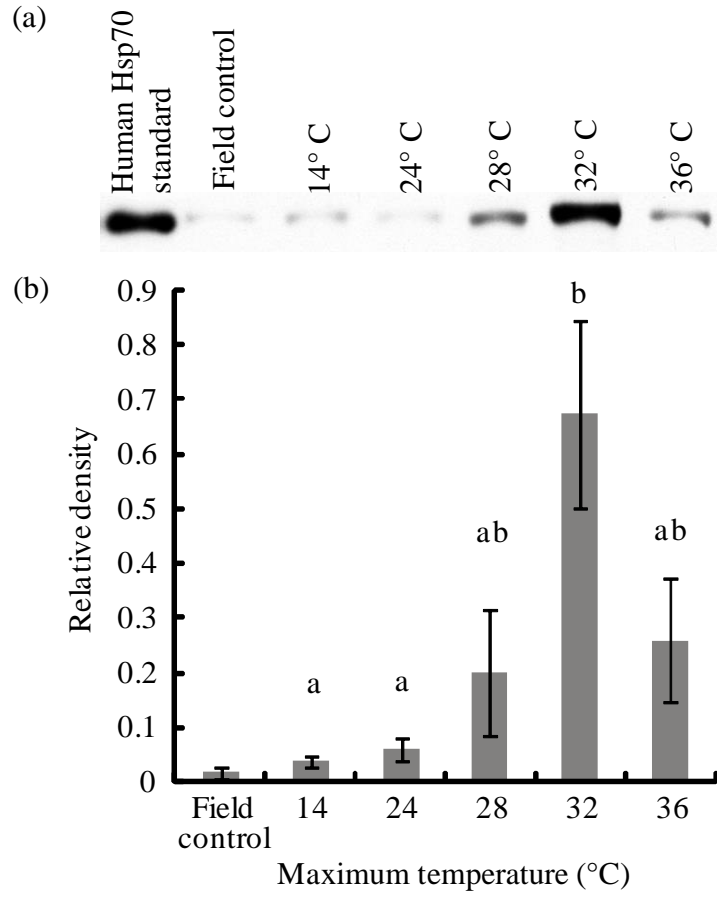
Fig. 1.



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Fig. 2.

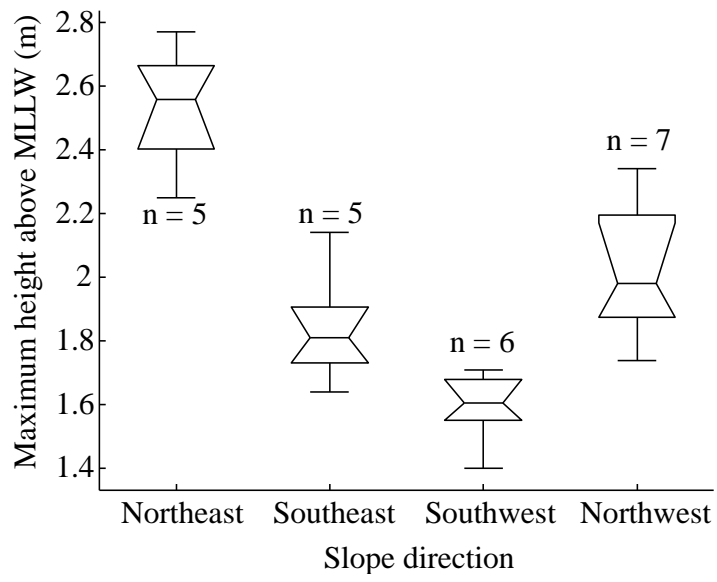


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Fig. 3.

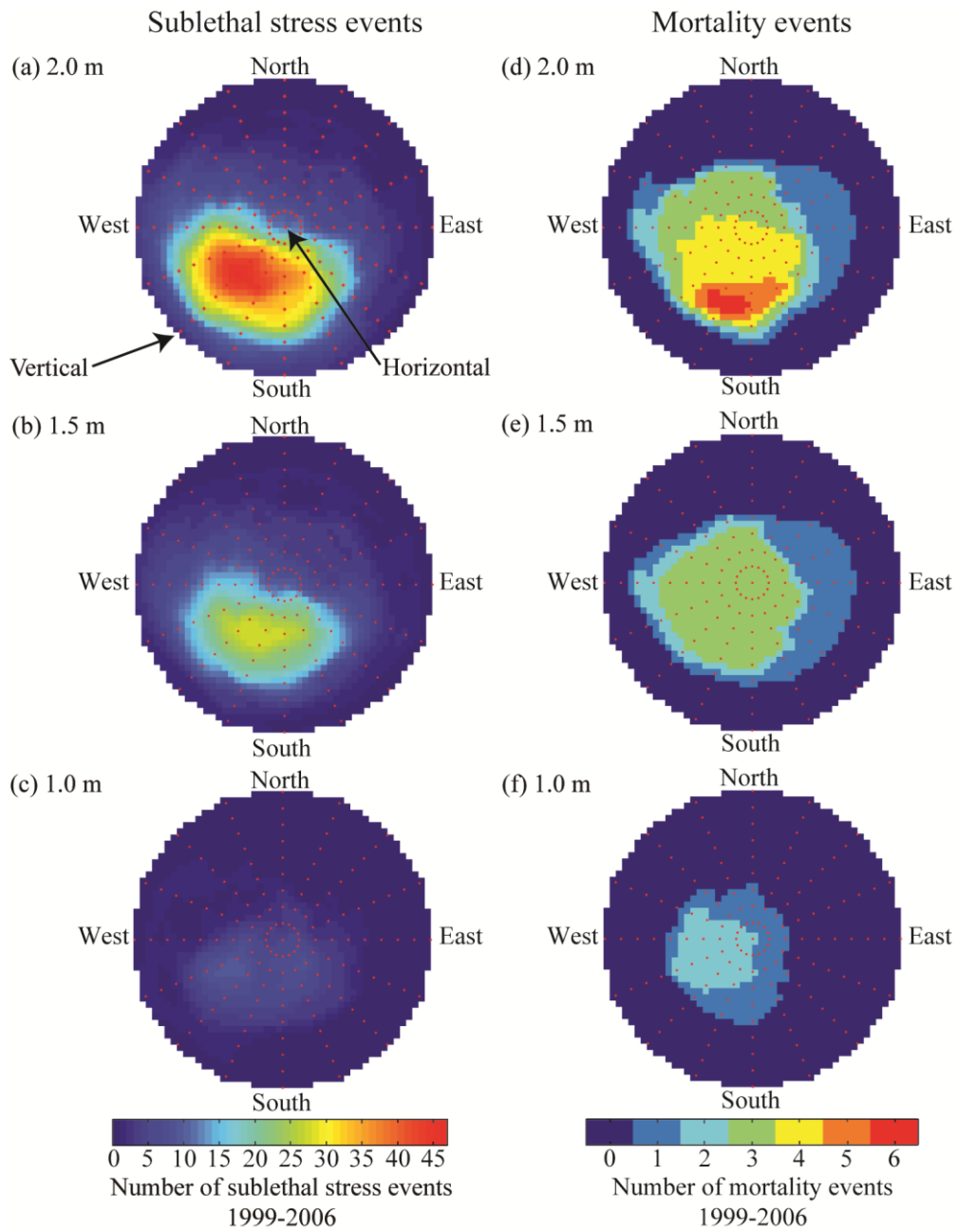
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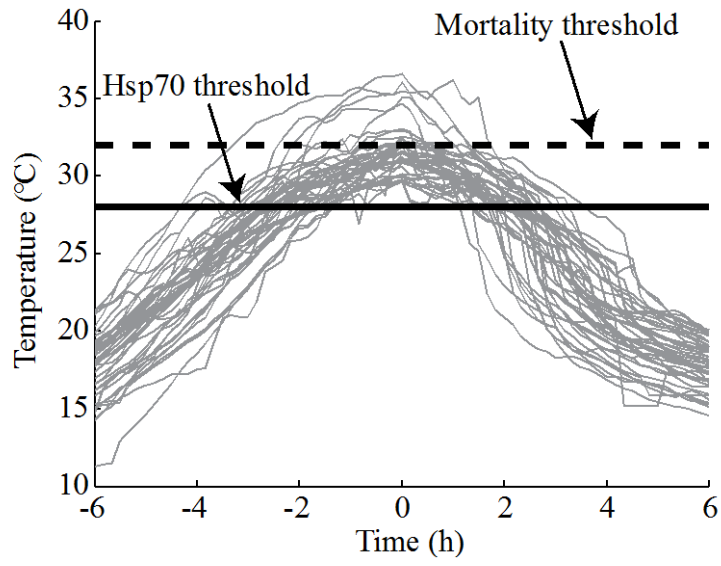
Fig. 4.



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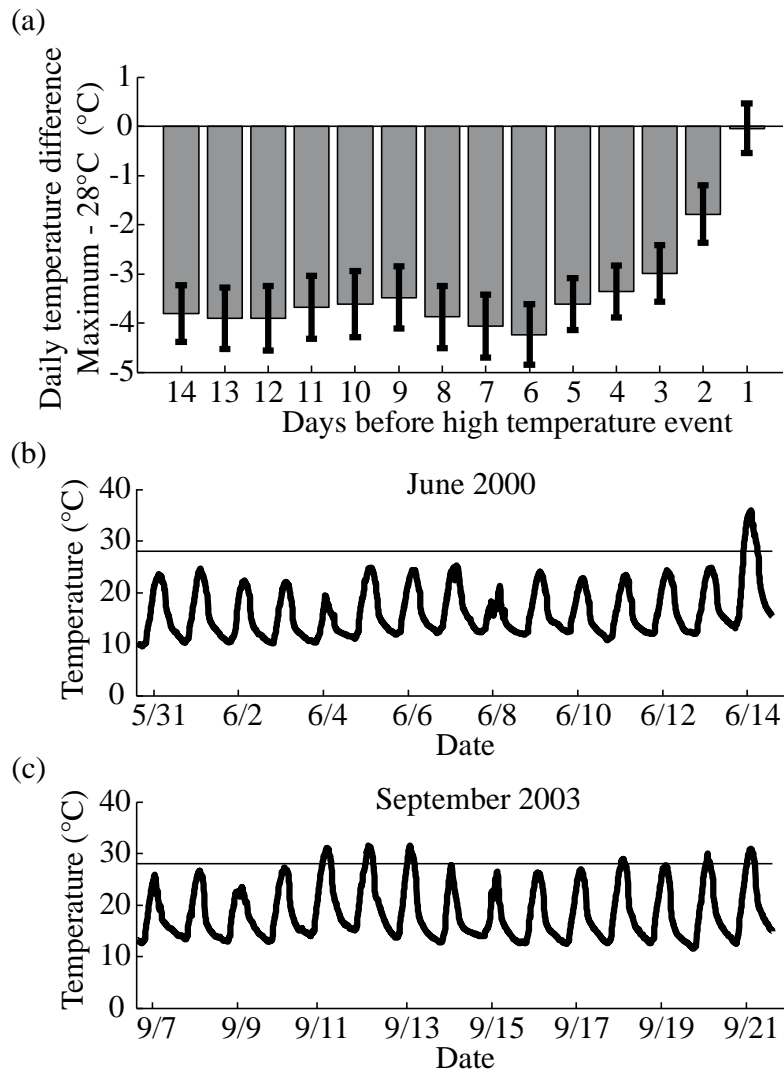
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Fig. 5.



782

783 **Fig. 6.**



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Fig. 7.

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